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Adipose tissue inflammation and coagulation in humans

by

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Stockholm 2010

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Published and printed by Larserics Digital print, Stockholm
Cover picture: Eva Sverremark-Ekström, photography
“The dark horse” -IHC staining of PAI-1 in adipose tissue after open heart surgery
Other paper-work Mattias Ekström

ISBN 978-91-7457-037-3

To Eva, Sofia and Edvin

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ABSTRACT

Background

Adipose tissue (AT) is not only a store of energy but an endocrine organ with capacity to produce and release proinflammatory mediators into the circulation. Obesity is an inflammatory disease, with increased circulating levels of interleukin (IL)-6, due to synthesis in AT. As current knowledge regarding AT inflammation, to a great extent relies on studies done in non-stimulated or chronic inflammatory conditions, it is important to add data from human studies, using different models of induced acute systemic inflammation. As obesity is becoming a global disease it is also an increasing risk factor for cardiovascular disease (CVD). CVD events are known complications after surgery and severe infection. The mechanisms behind this increased risk are still poorly understood but an acute systemic inflammation is a common denominator.

Methods and results

Study I: We investigated if a standardised systemic inflammation, induced by a vaccination against Salmonella typhi, would trigger inflammatory gene expression in AT. Healthy volunteers were investigated whereof half of them were vaccinated. Plasma levels of IL-6 increased 8 hrs after vaccination. In peripheral blood mononuclear cells we found an increased tumour necrosis factor gene expression after 4 hrs. In AT there were no differences in gene expression between the two groups.

Study II: Gene expression and production of inflammatory mediators in different AT depots were investigated after open heart surgery. Plasma levels of IL-6 increased 25-fold. In both omental and subcutaneous AT, we found a strong upregulation of nuclear factor-κB regulated genes. Immunohistochemistry (IHC) showed staining for E-selectin associated with a high number of macrophages in close contact with and in the vascular wall. Increased levels of IL-6 were detected in microdialysate from subcutaneous AT.

Study III: Plasminogen activator inhibitor-1 (PAI-1) synthesis in AT was studied after acute systemic inflammation, induced by open heart surgery. Gene expression of PAI-1 increased 27-fold in omental AT and 3-fold in subcutaneous AT. After surgery, IHC staining showed localization of PAI-1 antigen within endothelial cells, in the AT interstitium close to AT vessels and in solitary cells between the adipocytes. The upregulated gene expression and protein synthesis in AT was followed by increased concentrations of PAI-1 antigen in plasma.

Study IV: This was a sub-study of study I and II, with the aim to investigate the effects of an acute systemic inflammation on adiponectin and leptin synthesis. Neither plasma levels of adiponectin nor leptin were changed after vaccination. Gene expression of adiponectin and leptin were unaltered in both omental and subcutaneous AT after surgery.

Conclusion

Vaccination stimulates a mild systemic inflammation but does not trigger proinflammatory gene expression in AT. Open heart surgery induced a strong inflammatory response in both omental and subcutaneous AT including adhesion of macrophages to an activated endothelium and release of IL-6 from AT interstitium. We found no evidence that an acute systemic inflammation could affect synthesis of adiponectin or leptin indicating that these two adipokines are not key elements in the early acute-phase response. There was a markedly increased gene expression and protein synthesis of PAI-1 in human AT after open heart surgery. The increase was most prominent in omental AT. PAI-1 synthesis in AT, following acute systemic inflammation, may be the link between inflammation and impaired fibrinolysis that might explain the increased risk for myocardial infarction after surgery or infection.

SAMMANFATTNING

Bakgrund

Fettvävnad är inte bara en energidepå utan också ett endokrint organ med kapacitet att producera och frisätta proinflammatoriska markörer till cirkulationen. Fetma är en inflammatorisk sjukdom med kroniskt förhöjda nivåer av interleukin (IL) -6, associerat med en ökad produktion av IL-6 i fettvävnad. Kunskapen kring inflammation i fettvävnad är till stor del baserad på studier av icke stimulerad eller kronisk inflammation varför det är viktigt att tillföra data från humanstudier av olika modellsystem för stimulerad akut inflammation. Då fetma ökar i västvärlden är det också en växande riskfaktor för hjärt- och kärlsjukdom. Det finns också en ökad risk för akut hjärtinfarkt efter kirurgi och infektion. De bakomliggande mekanismerna till denna ökade risk är ofullständigt kända men en akut systemisk inflammation är en gemensam nämnare.

Metoder och resultat

Studie I: Vi undersökte om en standardiserad systemisk inflammation, stimulerad av en vaccination mot Salmonella typhi kunde aktivera inflammation lokalt i fettvävnad. Friska frivilliga försökspersoner undersöktes varav hälften blev vaccinerade. 8 timmar efter vaccination fann vi ökade nivåer av IL-6 i plasma. I perifera mononukleära vita blodkroppar fann vi ett ökat genuttryck av tumour necrosis factor efter 4 timmar. I fettvävnad fann vi ingen skillnad mellan grupperna avseende det proinflammatoriska genuttrycket.

Studie II: Här undersöktes genuttryck och produktion av proinflammatoriska markörer i underhudsfett och visceral fettvävnad, stimulerat av öppen hjärtkirurgi. Efter kirurgi fann vi 25 ggr ökade nivåer av IL-6 i plasma. Både i visceral fettvävnad och i underhudsfett fann vi ett starkt ökat genuttryck av gener reglerade av den signalväg som styrs av ”nuclear factor κB”. Färgning med immunohistokemi visade E-selectin tillsammans med ett stort antal makrofager i och intill kärlväggen. Vi fann även ökade nivåer av IL-6 i mikrodialysat från underhudsfett.

Studie III: Syntes av plasminogen activator inhibitor-1 (PAI-1) i fettvävnad studerades efter akut systemisk inflammation, stimulerad av öppen hjärtkirurgi. Genuttrycket av PAI-1 ökade 27 ggr i visceral fettvävnad och 3 ggr i underhudsfett. Efter kirurgi visade immunohistokemisk infärgning ett starkt uttryck av PAI-1 protein i endotelceller, mellan fettceller, i närheten av kärl och i enstaka celler mellan fettcellerna. Det ökade genuttrycket och proteinproduktionen i fettvävnad följdes sedan av ökade nivåer av PAI-1 i plasma.

Studie IV: I en sub-studie till studie I och II undersöktes effekterna på produktionen av adiponectin och leptin efter en akut systemisk inflammation. Varken plasmanivåer av adiponectin eller leptin förändrades efter vaccination. Genuttrycken av adiponectin och leptin förblev oförändrade i både visceral fettvävnad och underhudsfett efter öppen hjärtkirurgi.

Slutsats

Vaccination orsakar en mild systemisk inflammation men påverkar inte genuttrycken för proinflammatoriska markörer i fettvävnad. Öppen hjärtkirurgi resulterade i en omfattande inflammatorisk aktivitet i fettvävnad, såväl visceralt som i underhud, inkluderande adhesion av makrofager till aktiverat endotel i kärlväggen och frisättning av IL-6 från utrymmet mellan fettcellerna. Vi fann inga bevis på att en akut systemisk inflammation påverkade syntesen av adiponectin eller leptin vilket indikerar att dessa två adipokiner inte har en nyckel-roll tidigt under en akut-fas respons. Efter öppen hjärtkirurgi fann vi ett uttalat ökat genuttryck och proteinproduktion av PAI-1 i fettvävnad. Ökningen var mest uttalad i visceral fettvävnad. En ökad produktion av PAI-1 i fettvävnad kan vara länken mellan inflammation och försämrad fibrinolys som kan förklara den ökade risken för hjärtinfarkt efter kirurgi eller infektion.

List of Original Papers

This thesis is based on the following original studies which will be referred to by their Roman numerals.

I. **Ekström M**, Eriksson P, Tornvall P.
Vaccination, a human model of inflammation, activates systemic inflammation but does not trigger proinflammatory gene expression in adipose tissue.
J Intern Med. 2008 Dec;264(6):613-7

II. **Ekström M**, Halle M, Bjessmo S, Liska J, Kolak M, Fisher RM, Eriksson P, Tornvall P.
Systemic inflammation activates the nuclear factor- κB regulatory pathway in adipose tissue.
Am J Physiol Endocrinol Metab 2010;299:234-240

III. **Ekström M**, Liska J, Eriksson P, Sverremark-Ekström E, Tornvall P.
Stimulated *in vivo* synthesis of plasminogen activator inhibitor-1 in human adipose tissue
Submitted

IV. **Ekström M**, Söderberg S, Eriksson P, Tornvall P.
Acute systemic inflammation does not affect adiponectin and leptin synthesis in humans
Manuscript

List of Abbreviations

ACEi/ARB	angiotensin-concerting enzyme inhibitor/angiotensin receptor blocker
ACTH	adrenocorticotropic hormone
AT	adipose tissue
BMI	body mass index
CABG	coronary artery bypass grafting
CCL	chemokine ligand
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CPB	cardiopulmonary bypass
CRP	C - reactive protein
CV	coefficient of variation
CVD	cardiovascular disease
DAMP	damage-associated molecular pattern
ELISA	enzyme linked immunosorbent assay
GP	glycoprotein
H_2O_2	hydrogen superoxide
ICAM	intracellular adhesion molecule
IKK	IκB kinase
IL	interleukin
IL-6R	interleukin 6 receptor
INF	interferon
LDL	low density lipoprotein
LPS	lipopolysaccharide
mRNA	messenger ribonucleid acid
NEMO	NF-κB essential modulator
NF-κB	nuclear factor κB
NO	nitric oxide
O^-	superoxide anion
PAI-1	plasminogen activator inhibitor 1
PAMP	pathogen associated molecular pattern
PCI	percutaneous coronary intervention
RIA	radio immuno assay
ROS	reactive oxygen species
RQ	relative quantification
RT-PCR	real time polymerase chain reaction
SEM	standard error of mean
sIL-6R	soluble interleukin 6 receptor
TLR	toll like receptor
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
uPA	urokinase-type plasminogen activator
VCAM-1	vascular-cell adhesion molecule-1

INTRODUCTION

General background

Atherosclerosis commonly causes cardiovascular disease (CVD), a major morbidity worldwide and still the leading cause of mortality in US and Western Europe [1]. Conventional risk factors for developing CVD are hypertension [2], hypercholesterolaemia [3], diabetes mellitus [4] and smoking [2, 5]. Substantial effort has been made to reduce these risk factors and improve treatment of CVD which have resulted in decreased numbers of cardiovascular deaths [1]. In Sweden between 1986 and 2002, the mortality from coronary heart disease has decreased by 53.4% in men and 52.0% in women, mainly due to risk factor reductions [6]. Nevertheless, an increased prevalence of obesity has occurred during the last decades which is likely to undermine the important effort made to prevent CVD [7].

Obesity is the most common nutritional disorder in the industrialized world. Today, more than 50% of the adult population in Great Britain and US is overweight (body mass index (BMI)≥25) and approximately 30% is obese (BMI≥30) [8]. Twenty years ago, only 5% of adults in Sweden were obese. Today, around 50% of adult men and 40% of adult women are overweight and approximately 10% are obese in both sexes [9]. The excess of body fat has major consequences on western world morbidity. It is the most potent modifiable factor of the metabolic syndrome, characterized by abdominal fat distribution, high blood pressure, disturbed lipid metabolism and impaired glucose tolerance. Since obesity is becoming a global epidemic, it is also an increasingly important risk factor for CVD [10, 11] and actually, also an independent risk factor for developing myocardial infarction [12].
The common denominators of atherosclerosis and obesity extend beyond their overlapping incidence and their association to CVD risk factors. They also share common or similar pathophysiological pathways; atherosclerosis and obesity are today considered as chronic inflammatory processes characterized by activation of both innate and adaptive immunity [13].

Myocardial infarction and its trigger factors

Generally, myocardial infarctions result from coronary atherosclerosis with superimposed coronary thrombosis. Myocardial infarctions arise from ruptures of the fibrous cap, which is covering the atherosclerotic plaque and is followed by exposure of substances that promote platelet activation and thrombin formation [14]. Ultimately, this leads to a thrombus that interrupts blood flow which results in an imbalance between oxygen demand and supply resulting in myocardial necrosis.
Inflammation is crucial in the pathogenesis of plaque instability and therefore also important for the development of myocardial infarction. Plasma levels of inflammatory markers such as C-reactive protein (CRP) and interleukin (IL)-6 correlate with the clinical course and outcome of myocardial infarction [15]. There is a circadian variation of myocardial infarction with a higher incidence in the early morning which can be explained by a combination of sympathetic stress, hypercoagulability of the blood and activated platelets [16]. Physical activity or emotional stress, associated with increased vasoconstriction following sympathetic stress, might also trigger plaque disruption and coronary thrombosis [17].
Cardiovascular events are also known complications in up to five percent of patients undergoing non-cardiac surgery [18]. Furthermore, there is a five-fold increased risk of myocardial infarction

during the first week after a severe infection [19, 20]. The mechanisms behind this increased risk for myocardial infarction after surgery or infection are still poorly understood but acute systemic inflammation is a common denominator.

Atherosclerosis and plaque rupture

Development of atherosclerosis is initiated by activation and dysfunction of endothelial cells in individuals with signs of systemic inflammation and/or known risk factors for CVD. Dysfunctional endothelial cells cause leukocyte and platelet adhesion to damaged endothelium, Figure 1a. An increased permeability of the endothelial cells increases accumulation of lipid components, such as low density lipoprotein (LDL) to migrate into the subendothelial space. Monocytes, recruited to the intima become loaded with oxidized LDL and accumulate in the vessel wall and transform into foam cells which results in the formation of fatty streaks [21]. The development from fatty streaks to atherosclerotic plaques follows further accumulation of inflammatory cells and lipid components that is surrounded by smooth muscle cells and collagen-rich matrix. The inflammatory cells are represented by macrophages, mast cells and T-cells [22, 23], Figure 1b. Extended progression of the plaque results in a stenosis that narrows the artery lumen and affects the blood flow. The central core of the plaque can become necrotic and neovascularization may result in leaking of blood and haemorrhage which also contribute to plaque progression, Figure 1c. Normally, the atherosclerotic process takes many years to develop and over time, the plaque cells secrete matrix-degrading proteases and cytokines that results in a thinning of the fibrous cap that covers the plaque and prevents that pro-thrombotic materials from inside the plaque get in contact with circulating blood. In unstable plaques, a further thinning of the cap will make it to split up, causing plaque rupture. Plaque rupture, which is detectable in 60-70% of cases of acute myocardial infarctions [24] is dangerous because it exposes the pro-thrombotic contents from inside the plaque to circulating blood which results in activation of the coagulation system and finally a thrombosis in the artery. Ultimately, the thrombosis causes artery occlusion which prevents blood flow and results in a myocardial infarction, Figure 1d [15, 25].

Clinical studies as well as experiments on animal models and in cell cultures have all contributed to the understanding of the atherosclerotic process. Often our knowledge about pathophysiology on a molecular level rests on animal or *in vitro* experiments but of course these findings need to be reproduced, if possible with investigations on human tissues *in vivo*. Mice do not develop atherosclerosis under normal conditions but genetically modified animals with deletion of the gene encoding for apolipoprotein E (Apo-E knockout mice) develop hypercholesterolaemia and spontaneous atherosclerosis. Studies on hypercholesterolaemic mice have described that the infiltration of LDL into the arterial intima starts an inflammatory response [26]. Another interesting finding from mice models is that inhibition of platelet activation reduces leukocyte infiltration and atherosclerosis in Apo-E knockout mice [27]. However, this has not been described in humans.
In response to hypercholesterolaemia activated endothelial cells upregulate vascular-cell adhesion molecules 1 (VCAM-1) and circulating monocytes and lymphocytes that express VCAM-1 receptors on their surface, adhere to these sites on the endothelium. Once these circulating white blood cells are attached to the vessel wall, chemokines produced in the intima, stimulate them to migrate through the endothelial cell layer and into the subendothelial space. Furthermore, results from mice models have demonstrated that pharmacological inhibition of these chemokines and adhesion molecules blocks atherosclerosis [28, 29].

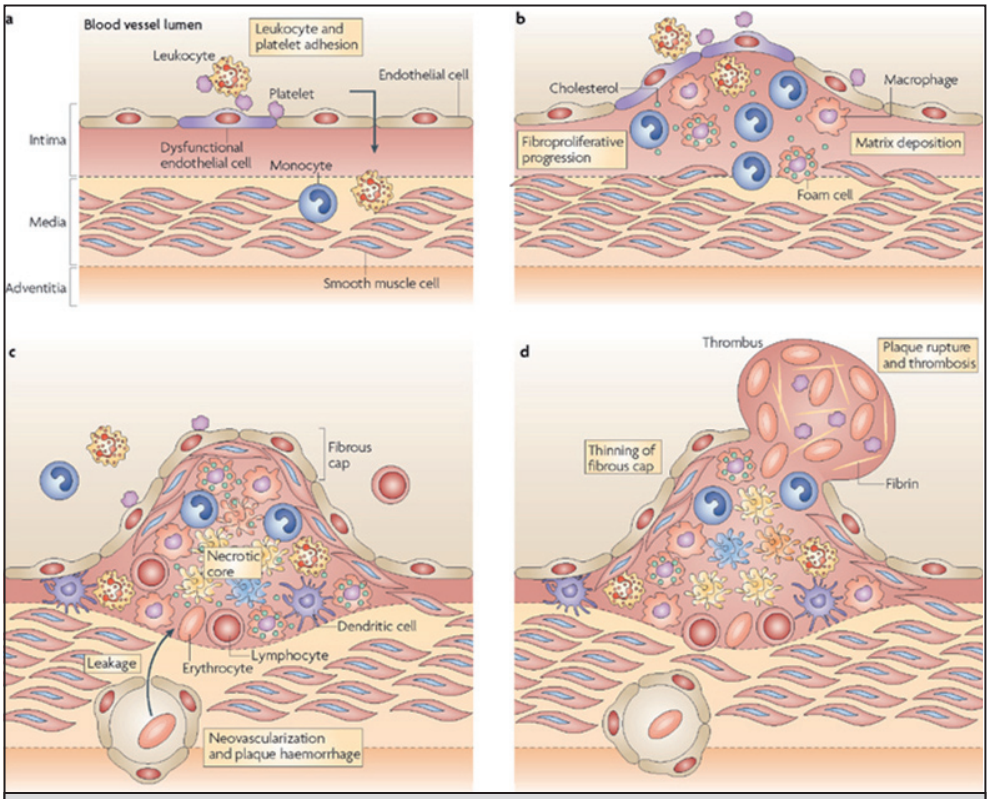

Figure 1. Evolution of atherosclerosis. **a)** Endothelial cell dysfunction and activation under pro-inflammatory conditions of hyperlipidaemia leads to early platelet aggregation and leukocyte adhesion and increased permeability of endothelium. **b)** Monocytes accumulate lipids and transform into macrophages or foam cells, which result in fatty streaks. **c)** Apoptosis of macrophages and smooth muscle cells creates a necrotic core, and a fibrous cap. Neovascularization within the plaque and from the adventitia can result in haemorrhage. **d)** Thinning of the fibrous cap, ultimately leads to plaque rupture with release of debris, activation of the coagulation system and plaque thrombosis of the artery. This results in arterial occlusion and myocardial infarction or stroke.
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Not only innate but also the adaptive immune response is central in atherosclerosis and T-cell infiltrates are always present in atherosclerotic lesions. Exposure of monocytes/macrophages to oxidized LDL results in T-cell activation [30]. The majority of these activated T-cells differentiate into Th1 effector cells which produce the macrophage activating cytokine interferon (INF) -γ. INF-γ activates macrophages and vascular cells resulting in vascular inflammation. The process is modulated by regulatory T-cells by producing anti-inflammatory cytokines. All these processes induce atherosclerosis and depletion of INF-γ has been shown to inhibit the development of atherosclerosis in animal studies [15].
Inflammatory cytokines such as tumour necrosis factor (TNF) and IL-1β are synthesized in atherosclerotic plaques and induce substantial production of IL-6. IL-6, in turn stimulates the

liver to produce amounts of acute phase reactants such as CRP. Although all these cytokines are important in each step of the pro-inflammatory response the upregulation of CRP makes it particularly useful as a clinical marker of atherosclerosis [31].

Adipose tissue and inflammation

The pro-inflammatory cytokines are also synthesized in adipose tissue (AT), thereby contributing to the low-grade chronic inflammation seen in obese individuals. Interestingly, in this way, there is a cross-talk between inflammation and metabolism.

Traditionally, AT is thought to merely represent a passive store of energy. However, recent research has proved AT to be a highly metabolically active organ, also related to several risk factors for atherosclerosis and CVD. Obesity has been shown to exhibit multiple manifestations of inflammation where AT is an endocrine organ with capacity to produce and release proinflammatory active mediators into the circulation.

Adiponectin and leptin are two adipokines primarily synthesized by adipocytes. Adiponectin has anti-inflammatory and anti-atherosclerotic activity [32]. Furthermore, adiponectin has important metabolic effects in obesity, including improvements on endothelial function and insulin resistance [33]. Consistent with these findings both obesity and CVD have been described with decreased circulating levels of adiponectin [34, 35].

Leptin was one of the first hormones isolated from AT and it plays a central role in the regulation of energy expenditure. Knock-out mouse models lacking the gene encoding for leptin have been demonstrated to have increased BMI. The supply of leptin to these mice decreased their intake of calories [36]. Leptin regulates food intake through signalling in hypothalamic centres of the brain and leptin has now been established as an AT produced hormone with major impact on BMI, energy regulation and insulin resistance [37]. In addition, leptin has been associated with the inflammatory markers IL-6 and CRP indicating an interaction with the acute inflammatory response [38].

AT generates an inflammatory setting, characterized by high levels of CRP and other important inflammatory bio-markers, e.g proinflammatory cytokines and adhesion molecules. Obesity has been associated with increased unstimulated levels of TNF, IL-6 and vascular adhesion molecules, such as VCAM-1 [39, 40]. TNF is synthesized by adipocytes and might have paracrine effects on AT. Moreover, local synthesis of TNF by adipocytes is elevated in obese subjects [39, 40]. The association between AT and inflammation has further been elucidated by demonstrating an *in vivo* release of IL-6 from human AT [41] and it has been estimated that approximately 30% of total circulating IL-6 is produced by AT [42]. The AT inflammatory milieu is further demonstrated by increased levels of IL-6 in the circulation, due to increased expression and synthesis of proinflammatory cytokines in AT [13, 43, 44]. Also, increased gene expression of CRP has been found in AT in patients with chronic inflammatory diseases [45].

Interestingly, a prospective study has demonstrated a marked reduction in circulating levels of CRP in patients undergoing weight loss surgery [46] and weight loss is also associated to decreased AT macrophage infiltration [47]. Furthermore, weight reduction in obese subjects resulted in decreased levels of inflammatory cytokines and adhesion molecules why it appears that weight loss also reduce CVD risk [48, 49].

Ex vivo experiments using human cultured adipocytes stimulated by lipopolysaccharides (LPS), have demonstrated an induction of the nuclear factor κB (NF-κB) regulatory pathway [50, 51]. Furthermore, LPS has also been found to induce AT inflammation *in vivo* demonstrated by increased gene expression of inflammatory mediators in subcutaneous AT [52-54]. However,

since current knowledge about acute AT inflammation mainly rests upon *ex vivo* studies or LPS-stimulated subcutaneous AT inflammation, studied on a gene expression level, further *in vivo* studies including other stimuli of inflammation with a focus on both gene expression and protein production in omental and subcutaneous AT, are needed to get a better understanding of how AT inflammation is activated in response to an acute systemic inflammation.

Innate immunity

A successful immune response relies on complex interactions between different immune cells. These interactions are mediated by a group of soluble peptides called cytokines. The term interleukin (IL) is used for cytokines that are secreted by, and act on leukocytes. The chemokine family consist of peptides important for the regulation of leukocyte trafficking and the migratory behaviour of leukocytes into subendothelial tissues. Other chemokines are needed for adhesion and leukocyte activation.
Traditionally the immune system has been divided into adaptive and innate components. Adaptive immunity is mainly structured around two classes of specialized lymphocytes, B-cells and T-cells. Clonal expansion of lymphocytes in response to an infection is necessary for an efficient immune response. However, it takes three to five days to produce sufficient numbers of clones which can differentiate into effector cells for specific targets and to develop an immunological memory which recognizes and prevents a later infection with the same microorganism. This delay leaves time enough for pathogens to damage the host.
In contrast, the rapid mechanisms of innate immunity are activated immediately, within four hrs after infection or tissue injury hereby making it possible to quickly inhibit and control the invading pathogen [55, 56].
In the next pages I will focus on presenting immune cells, cytokines and chemokines that are relevant, to my studies.

Innate immunity refers to the first line of defence that limits infectious challenge in the very early stages after pathogen exposure. The ability to recognize and limit infectious challenge is mediated by pre-existing molecular and cellular mechanisms that distinguish common and frequently encountered pathogens. There are different anatomical levels where the innate immune components act to protect against pathogen invasion. For instance, the first line of defence is the barrier that consists of epithelial cells in skin and mucosa which serves as an effective wall against pathogenic microbes. If the pathogens cross this barrier they are efficiently removed and destroyed by immune cells that are operating in underlying tissues. One of the most ancient immune defence line, the ability to produce antimicrobial peptides, lies within this part of innate immunity, actually found both in plants and animals and therefore predate the separation of these two evolutionary lineages [57]. Macrophages and neutrophils produce toxic products that efficiently kill microbes. Most important of these are nitric oxide (NO). Moreover, these cell types also transform O^2 into highly reactive forms, called reactive oxygen species (ROS), such as superoxide anion (O^-) and hydrogen superoxide (H_2O_2) [58]. ROS, in turn, is able to activate the NF-κB signalling pathway [59].
Innate immunity lies behind most inflammatory responses through interactions with the targets of innate immune recognition, which are conserved molecular patterns, called pathogen-associated molecular patterns (PAMPs), such as LPS or damage-associated molecular patterns (DAMPs). The acute inflammatory response is triggered in the first instance by the innate immune receptors of macrophages, polymorphonuclear leukocytes and mast cells. The adaptive immune system completes the innate immune response with specific recognition

of proteins, carbohydrates, lipids or nucleic acids, using the same activated effector cells as were generated during the innate immune response. In this way the two systems are linked to each other [55, 60].

Rolling and extravasation of leukocytes

The recruitment of leukocytes is one of the most important steps of innate immunity. This process is mediated by cell-adhesion molecules, expressed on the surface of endothelial cells at the site of infection or inflammation in local blood vessels, mainly veins. The selectins are induced on activated endothelium and initiate interactions with leukocytes by binding to ligands on the surface of circulating leukocytes. This reaction does not anchor the leukocytes to the vessel wall but makes them rolling along the endothelium. Next, the rolling leukocytes bind to intercellular adhesion molecules (ICAMs) on the endothelial surface, resulting in a tighter adhesion to the vessel wall. The activation of endothelium is driven by interactions between cytokines produced by macrophages, in particular TNF and a release of preformed P-selectin that will be expressed on the endothelial surface. In addition, mRNA encoding for E-selectin is upregulated and within two hrs the endothelial cells are expressing mainly E-selectin on their surface [61].

When the leukocytes have stopped their rolling movement along the endothelium, further interactions with integrins, allows them to squeeze between the endothelial cells into the subendothelial tissue. Finally the leukocyte migrates along the gradient of chemokines, such as chemokine ligand (CCL)-2 and IL-8/CXCL8, Figure 2.

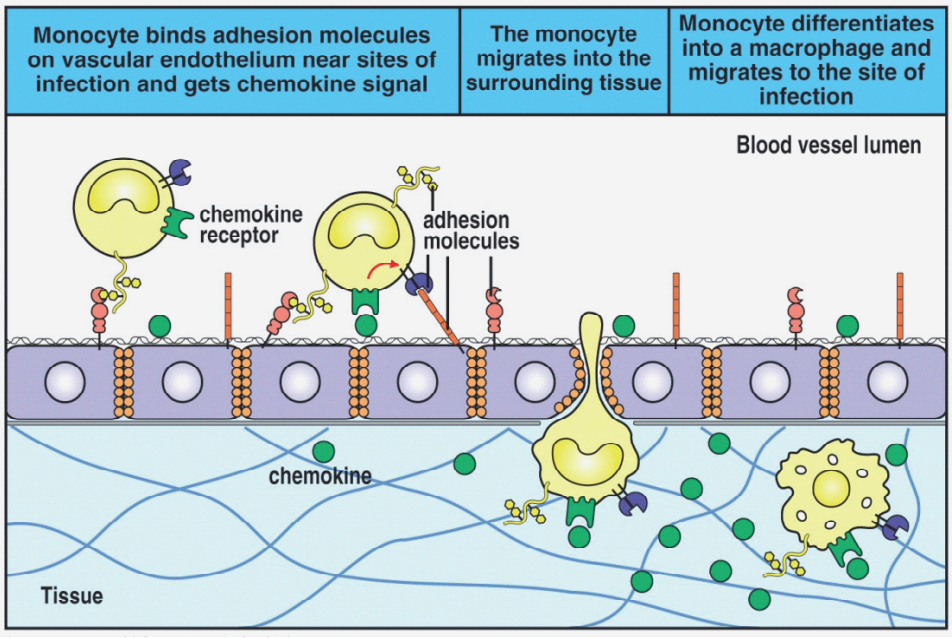

Figure 2. Monocytes circulating in the blood vessel (mainly veins) recognize blood vessel walls near the site of inflammation and leave the bloodstream, to migrate into the tissue toward the site of infection or inflammation.

CCL-2, primarily acts on monocytes and induce their differentiation to macrophages on their migration from circulating blood to the site of infection and inflammation [62]. CXCL8 was the first chemokine to be described and has similar functions as CCL-2 but act more specifically on neutrophils by attracting them to leave the blood stream [63].
In the subendothelial space the pathogen is recognized and eliminated by mononuclear phagocytes, the macrophages that are essential cells in the immune system. Macrophages differentiate continuously from monocytes that leave the circulation to migrate into the surrounding tissue, as described above. They are able to recognize pathogens through their surface receptors and discriminate between self from non-self. One of these receptors is the cluster of differentiation (CD)14, a receptor that binds to LPS and is predominantly found on monocytes and macrophages. The LPS: CD14 complex in turn, triggers the membrane protein toll like receptor (TLR)4 which activates the nuclear factor (NF) κB signalling pathway resulting in production of pro-inflammatory cytokines and chemokines [56, 64].

NF-κB signalling pathway

It is well established that activation of the NF-κB signalling pathway is essential for regulating immune responses and is implicated in many inflammatory diseases. However, inhibition of this important regulatory pathway is not a solution or even a possible way to treat inflammatory diseases. In mouse models, total inhibition of NF-κB in non-immune epithelial cells results in severe and mortal inflammatory conditions [65, 66]. This, apparently paradoxical finding, focus on the multi-disciplinary roles of NF-κB signalling and suggests that inhibition of NF-κB in certain tissues might have pro-inflammatory effects by disrupting the physiological immune balance [64].
The NF-κB dimers consist of a complex of RELA (also known as p65) and p50. In resting cells, the NF-κB complex is associated with inhibitory peptides and is sequestered in the cytoplasm. Activation of NF-κB is controlled by the IκB kinase (IKK) complex and NEMO, a regulatory subunit (NF-κB-essential modulator). Pro-inflammatory signals stimulate receptors of the tumour necrosis factor receptor (TNFR) or IL-1/Toll-like receptor (TLR) super families, which activate the IκB kinase (IKK) complex. IKK, in turn phosphorylates IκB which starts a polyubiquitylation and a subsiding degradation of IκB. Hereafter, when IκB is removed, the NF-κB dimer (p50 and p65) is allowed to migrate into, and to accumulate in the cell nucleus where it binds to target genes and activates transcription. In this way, NF-κB is able to translate upstream signals into a rapid onset of gene expression. Mainly, NF-κB regulates genes with pro-inflammatory effects but also genes encoding for inhibition of apoptosis. These molecules are central in the innate immune response to fight invading pathogens and are required for migration of inflammatory cells to tissue areas where the NF-κB has been activated in response to infection or injury, Figure 3 [64].

IL-1β, IL-6 and TNF

IL-1β, IL-6 and TNF are pro-inflammatory cytokines controlled through the NF-κB regulatory pathway. They are characterized as being endogenous pyrogenes as they derive from endogenous sources in contrast to exogenous pyrogenes such as LPS that derives from bacterial components. They act on hypothalamus to alter the body temperature, and on muscles and AT to mobilize energy to increase body temperature. In conditions with fever both bacterial and viral replication are decreased whereas the adaptive immune system operates more efficiently. One of the most important effects of these three cytokines is the initiation of an acute-phase response which follows the action of IL-1β, IL-6 and TNF on hepatocytes. During an acute-phase response levels of some plasma proteins increase markedly and one crucial acute-phase

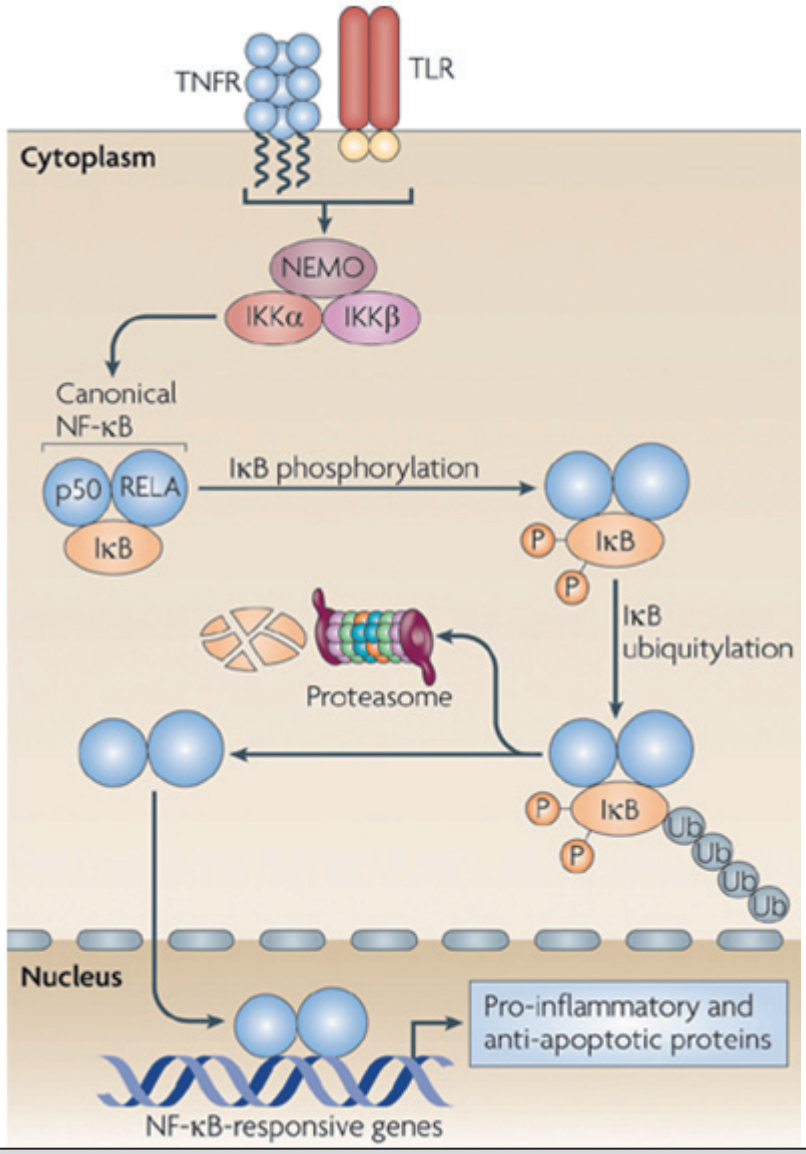

Figure 3. Schematic description of the classical NF-κB signalling pathway.
IkB kinase - IKK; NF-κB essential modulator - NEMO; Nuclear factor-κB - NF-κB; Toll-like receptor - TLR; Tumour necrosis factor receptor - TNFR;
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protein is CRP, a member of the pentraxin protein family which bind to certain bacteria and fungal cell walls. By binding to invading pathogens it enables phagocytosis by opsonization but it also activates the component cascade, another important part of the innate immune system that is not going to be further described in detail here.

IL-1β is produced by several cell types including monocytes, macrophages, dendritic cells and epithelial cells. Mediators that initiate IL-1β synthesis are PAMPs and pro-inflammatory cytokines including TNF. It also activates bone marrow epithelium to release neutrophils. The signals mediated by IL-1β result in down stream up-regulation of genes encoding pro-inflammatory chemokines and cytokines.

IL-6 is a multifunctional cytokine with an important role in host defence but its role as an inflammatory cytokine depends on whether there is an ongoing acute immune response or not. It is involved in the development of cells and tissues as well as in different pathological conditions [67, 68]. IL-6 is not constitutively produced but can be synthesized in response to inflammatory stimuli such as IL-1β, LPS and TNF [69, 70]. Glucocorticoids, in turn, suppress IL-6 expression in a variety of cell types [71]. However during stress and acute inflammation, glucocorticoids can also synergize with IL-6 and induce hepatocytes to produce acute phase proteins [72].
Furthermore, IL-6 has been shown to be released in significant amounts from skeletal muscle during exercise [73, 74]. Interestingly, the IL-6 derived from muscle during exercise differs from the cytokine response to sepsis with regard to TNF, since the cytokine response to exercise is not preceded by an increase in plasma TNF [74]. Monocytes/macrophages are not involved in the IL-6 production in skeletal muscles since no upregulation of IL1β, IL-6 or TNF gene expression has been demonstrated in these cells during exercise [75]. The biological importance of IL-6 synthesis in skeletal muscles during exercise might have other but immunological functions. One theory is that it acts as an endocrine signal indicating that the muscle glycogen store has reached critically low levels.
Moreover, IL-6 may have beneficial effects on the metabolism of carbohydrates and fat as well as exercise capacity [74]. Experiments on animals have demonstrated a preventing mechanism on obesity by IL-6 [76]. This mechanism is not fully understood but IL-6 might have effects on leptin sensitivity. Then the site of action would be in the hypothalamus where it stimulates the synthesis of adrenocorticotropic hormone (ACTH), thereby activating a negative feedback loop of inflammation and a link to the neuroendocrine system [76, 77].
IL-6 is produced by many different cell types including adipocytes, endothelial cells, fibroblasts, monocytes/macrophages and myocytes. In an immune response IL-6 regulates production of adhesion molecules involved in the release of other cytokines and induces the hepatic synthesis of CRP, as mentioned above. *In vivo* release of IL-6 from human AT has been demonstrated [41] and in addition, positive correlations between both circulating and AT levels of IL-6, TNF and serum CRP levels have been shown [44]. The various effects of IL-6 are mediated through a receptor complex consisting of a glycoprotein (gp130) and its transmambrane receptor (IL-6R) or its soluble receptor (sIL-6R) [78, 79]. The gp130 has been found to function as a receptor component for several cytokines other than IL-6. It can be found in almost all tissues and cells which explain the pleiotropy of IL-6. IL-6R is mainly expressed on hepatocytes and different subsets of leukocytes.

Recent research provides growing evidence of a pathological role of IL-6 in various diseases, such as inflammatory or auto-immune diseases. Based on these findings, a new therapeutic approach to block IL-6 with monoclonal antibodies against IL-6R in rheumatoid arthritis has been developed [67].

TNF is synthesized by cells of the immune system and is a strong mediator of inflammatory

and immune functions [68]. Macrophages in AT can produce TNF shown to be associated to proinflammatory activity that may contribute to atherosclerosis [37]. Circulating levels of TNF are usually very low during healthy conditions but increase rapidly during an infection or an inflammation [80]. TNF launches several downstream pro-inflammatory effects and mediates the expression and synthesis of adhesion molecules as E-selectin on vascular endothelium which together with chemokines contribute to the recruitment of leukocytes to the site of infection or inflammation. However, uncontrolled this crucial chemokine can cause vasodilatation and increased vascular permeability, leading to loss of circulating plasma volume and eventually to shock during sepsis.

Fibrinolysis and plasminogen activator inhibitor-1

Fibrinolysis is a cascade of enzymatic processes leading to degradation of fibrin. This process is determined by both plasminogen activators and inhibitors, whereof plasminogen activator inhibitor (PAI) 1 is believed to be the most important inhibitor. PAI-1 decreases fibrinolytic activity by acting as an inhibitor of both urokinase-type plasminogen activator (uPA) and tissue plasminogen activator (tPA). Inhibition of plasmin by PAI-1 results in inadequate fibrinolytic activity which may result in the formation of a vascular thrombosis. Hepatocytes, platelets and vascular endothelial cells are believed to be the main producers of PAI-1, but the contribution of different tissues to circulating PAI-1 may differ in health and disease [81]. Furthermore, there is a diurnal variation of plasma levels of PAI-1 with the highest levels seen at 6 am in the morning [82].

Increased plasma concentrations of PAI-1 are associated with an increased risk of deep vein thrombosis, pulmonary embolism and myocardial infarction [83-85]. Previously, an association between adiposity and impaired fibrinolysis has been observed [86-88] and obese diabetic subjects are reported to have increased circulating concentrations of PAI-1 [89]. Importantly, both murine and human adipocytes have been shown to express PAI-1 mRNA [88, 90, 91].

Other conditions associated with elevated levels of PAI-1 are physical inactivity, hyperlipidaemia (especially hypertriglyceridaemia) and the metabolic syndrome [92, 93]. PAI-1 is described as an acute phase protein and increases during infections and acute inflammations and high plasma levels during sepsis is associated to poorer prognosis [85]. The regulation of gene expression of PAI-1 in AT has been investigated in numerous studies. Proinflammatory cytokines, such as IL-1β and TNF increase PAI-1 mRNA in AT in animal models [90, 94, 95] while TNF, IL-1b and IL-6 all stimulate upregulation of PAI-1 gene expression in human adipocytes *ex vivo* [96, 97]. Other well-known inducers of PAI-1 synthesis in AT are angiotensin II, corticosteroids and insulin whereas catecholamines suppress PAI-1 gene expression and synthesis in AT [98-100]. The current knowledge regarding regulation of PAI-1 in AT is based on animal studies or *ex vivo* experiments on human adipocytes. However, it is unclear whether human adipocytes cultured *ex vivo* adequately represent the situation on a tissue level in vivo. So far, no study has described stimulated gene expression and protein synthesis of PAI-1 *in vivo* in human AT (Figure 4).

In vivo models of induced inflammation in humans

To investigate the effects of an acute systemic inflammation on different organs and tissues in humans, several models of induced inflammation have been used, Figure 5.

One way to stimulate a mild but systemic inflammation is the use of a commercially vaccine against Salmonella typhi, previously shown to cause endothelial dysfunction and activate coagulation [101-103]. Furthermore, this model has been used to demonstrate protective actions

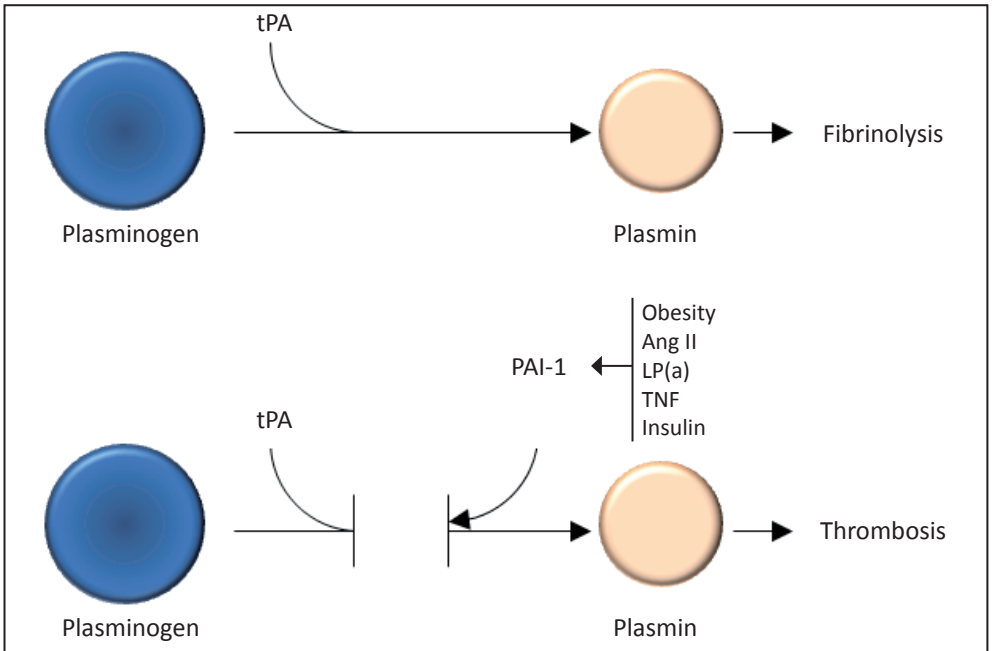

Figure 4. The normal balance between thrombosis and fibrinolysis is determined by tissue plasminogen activators (tPA) and their inhibitors. In obesity there is a relative increase in the levels of plasminogen activator inihibitor-1 (PAI-1) which promotes a prothrombotic state.
Ang II – angiotensin II; lp(a) – lipoprotein (a); TNF – tumour necrosis factor.

of aspirin on inflammation-induced endothelial dysfunction [104]. Vaccination is a safe model without any major side effects and results in 2-4-fold increased plasma levels of IL-6 after 8 hrs [101, 105]. The clinical relevance of vaccination has been discussed in the context of infection or inflammation as a trigger of myocardial infarction, but there has not been found any increased risk of a myocardial infarction after influenza, tetanus or pneumococcal vaccinations [19].

Previously, a mild but systemic inflammatory response has been reported after a percutaneous coronary intervention (PCI) according to a standard procedure. PCI results in doubled plasma levels of IL-6 after six hrs [106]. In addition, this inflammatory response is of clinical relevance since the increase in CRP is associated with the risk of new coronary events [107].

Another, non drug-induced experimental model of inflammation is periodontal therapy, where the mechanical removal of the biofilm and subgingival calculus deposits in order to reduce bacterial load and local inflammation, results in a 3-4-fold increase in plasma levels of IL-6 one day after therapy. Further, it causes endothelial dysfunction and has procoagulative effects [108-110].

Open heart surgery with cardiopulmonary bypass (CPB) is one of the strongest models of induced inflammation found in daily clinic. Open heart surgery results in an extensive acute systemic inflammation with a 30-40-fold increase in plasma levels of IL-6, 2-6 hrs after

start of surgery [111, 112]. Moreover, open heart surgery results in increased gene expression of inflammatory mediators such as IL-1β, TNF, TLR-2 and 4 in circulating leukocytes, possibly due to contact of circulating blood with the synthetic surface of the CPB system [113, 114]. The activation of the acute phase response due to open heart surgery and CPB is a complex process. Possibly there are different triggers: the surgical trauma itself, blood contact with non physiological surfaces as mentioned above, endotoxemia and ischemia [115]. This inflammatory response may contribute to several postoperative complications including myocardial dysfunction, bleeding disorders, respiratory failure and multiple organ failure.

To my knowledge, injection of a single bolus dose of endotoxin or LPS is the strongest experimental model of inflammation. This model has been used in animal and human studies for many years to investigate the relationships between infection and the acute-phase response in the host [116-118]. A bolus injection of LPS (2 ng/kg) results in a 300-fold increase in plasma levels of IL-6 2 hrs after LPS challenge [119]. The expected side-effects due to LPS are mild and transient flu-like symptoms.

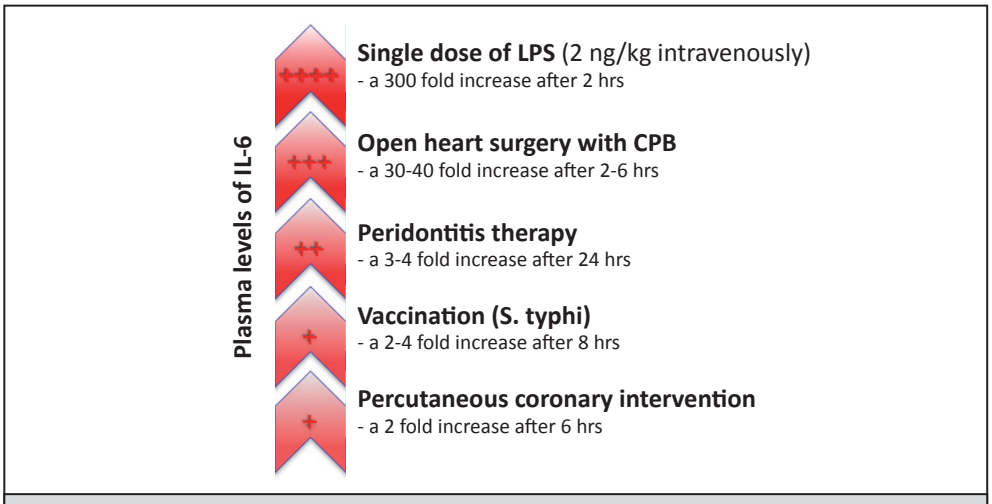

Figure 5. *In vivo* models of induced systemic inflammation in humans, and their effects on plasma levels of IL-6.
CPB – cardiopulmonary bypass, IL-6 – interleukin-6: LPS – lipopolysaccharide; S. typhi – Salmonella typhi.

Aims

The overall purpose of this thesis was to further investigate and to better understand if and how AT inflammation is initiated following an acute systemic inflammation in humans. In addition, potential mechanisms behind the increased risk of a myocardial infarction following an acute systemic inflammation have been investigated.

The major hypothesis is that an acute systemic inflammation in humans induces inflammatory activity in AT *in vivo*. A second hypothesis is that an acute systemic inflammation, through the activation of AT inflammatory capacity, induces gene expression and protein production of PAI-1 in AT.
The specific aims are:

I. - to investigate if a standardised inflammatory stimulus, using vaccination against S. typhi as a model of inflammation, would trigger inflammatory gene expression in AT.

II. - to analyze the *in vivo* gene expression and production of inflammatory mediators, focusing on innate immunity, in both omental and subcutaneous AT in patients undergoing open heart surgery with cardiopulmonary bypass.

III. - to study the plasminogen activator inhibitor-1 synthesis in AT stimulated by acute systemic inflammation, induced by open heart surgery.

IV. - to examine the effects of an acute systemic inflammation, induced by vaccination against S. typhi or open heart surgery respectively, on adiponectin and leptin synthesis.

MATERIALS AND METHODS

Study subjects

In this thesis two study groups have been investigated: the vaccination study group and the open heart surgery study group. The distribution of the subjects from these two groups between paper I-IV is described in detail in Figure 6a.

The investigations were made in accordance with the declaration of Helsinki and all subjects gave informed written consent to participate in the studies which were approved by the Ethics Committee of the Karolinska Institutet.

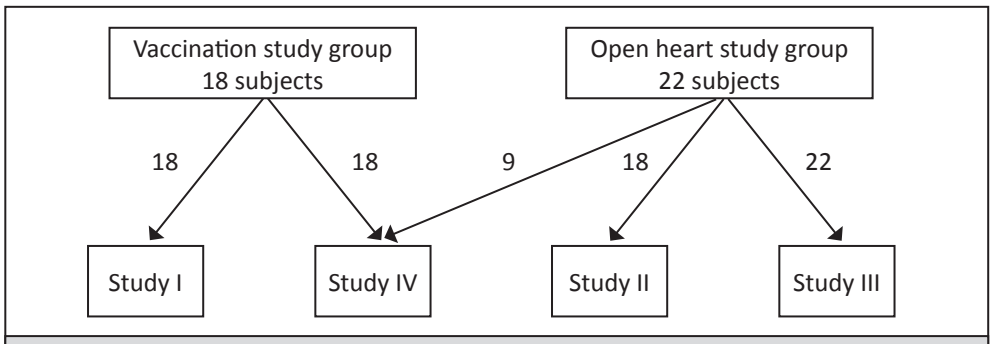

Figure 6a. The distribution of subjects between *paper I-IV.*

The vaccination study group (paper I and IV)

The vaccination study group comprised of eighteen healthy volunteers (16 men and 2 post-menopausal women) and all subjects were included and examined from May 2006 until June 2007. They were invited as being participants in two previous studies. One study comprised 387 healthy individuals that served as controls to patients with a first myocardial infarction before the age of 60 years, in order to investigate novel risk factors for atherosclerosis and myocardial infarction [120]. The other study included 96 healthy men in which postprandial triglyceridemia were studied in relation to intima-media thickness [121]. All together, 74 individuals were asked to participate but 56 were excluded after a telephone interview because of chronic medical conditions or unwillingness to participate. Further exclusion criteria were on-going treatment with anti-inflammatory drugs and a history of previous vaccination against S. typhi. Basic characteristics and study protocol are described in detail in *paper I.*

In brief, venous blood samples were obtained after 0, 4, 8, 12 and 24 hrs. After the initial blood sample, subjects in the vaccine group received a subcutaneous injection with vaccine against S. Typhi. Four hours after the first blood sampling all subjects underwent a subcutaneous fat biopsy from the periumbilical area of the abdomen [122] for gene expression studies. At 0 and 4 hrs venous blood samples were obtained for analysis of gene expression in PBMCs.

To control for differences in inflammatory responses due to different genotypes, all subjects in the vaccination study group were homozygous for the common -174 G allele in the -174G>C polymorphism in the IL-6 gene.

The open heart surgery group (paper II, III and IV)

Patients were eligible if they were planned for elective coronary artery bypass grafting (CABG) surgery and/or aortic or mitral valve replacement therapy according to a standard surgical procedure from May 2008 until December 2009 at the Department of Thoracic Surgery at the Karolinska University Hospital, Solna, Sweden. Patients were excluded if they had unstable coronary artery disease or were treated with corticosteroids. Twenty-two male patients who were planned for open heart surgery underwent blood sampling and/or AT biopsies for gene expression and/or immunohistochemistry before and after CPB.
Basic characteristics and study protocols are described in detail in *paper II and III*.

Methods

All methods are described in detail in each paper. Here follow a brief description of the different laboratory methods used.

Plasma analyses

Enzyme-linked immunosorbent assay (ELISA)

Plasma levels of IL-6 in *paper I and IV* were determined in duplicates using one high sensitive ELISA plate (R&D Systems) with an intra-assay coefficient of variation (CV) of 9.5%.
Plasma levels of IL-6 in *paper II and III* were analyzed in duplicates using one Quantikine Human IL-6 ELISA plate (R&D Systems) with an intra-assay CV of 10.2%.
Plasma levels of PAI-1 antigen in *paper III* were analysed in duplicates using the DuoSet ELISA for human Serpine E1/PAI-1 (R&D Systems). Mean intra-assay, respectively inter-assay CV was 6.5% and 5.1%.

Radio immuno assay (RIA)

Plasma levels of adiponectin and leptin in *paper IV* were analyzed in duplicates using double-antibody radio immuno assays (RIA) (Linco). CV for adiponectin was 15.2% at low (2–4 µg/mL) and 8.8% at high (26–54 µg/mL) levels. CV for leptin was 4.7% at both low (2–4 ng/mL) and high (10–15 ng/mL) levels.

Adipose tissue biopsies

In *paper I and IV* all subjects underwent a subcutaneous AT biopsy from the periumbilical area of the abdomen, as described [122]. The biopsy of 300-500 mg was washed in saline and immediately frozen in RNAlater (Ambion) and stored in – 80 C for gene expression studies. In the open heart surgery group paired AT biopsies of approximately 1 cm^3 were taken from all together 13 patients. The first 9 patients were investigated in *paper II and IV* where after 4 more patients were included for *paper III*. Both omental and subcutaneous AT biopsies were collected from six patients, only omental AT biopsies from one patient and only subcutaneous AT biopsies from six patients, see Figure 6b. The AT biopsies were collected before institution of CPB and at 15-20 min after removal of the aortic cross-clamp when the patient had been weaned off CPB. The omental AT biopsies were taken through a small opening to the abdomen in the bottom of the wound and the subcutaneous AT biopsies were taken deeply from the side of the median sternotomy incision.

RNA extraction and cDNA preparation

Total RNA was extracted from PBMCs in *paper I* using QIAamp RNA Blood Mini Kit

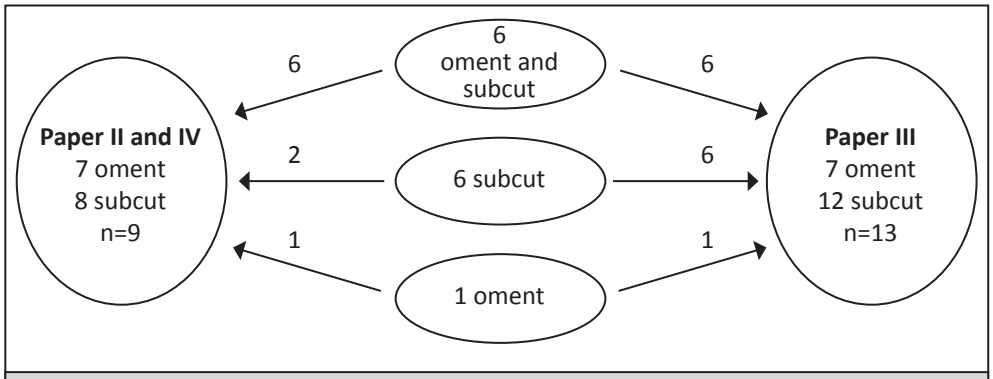

Figure 6b. The distribution of biopsies from omental and subcutaneous adipose tissue between *paper II-IV.*

(QIAGEN). Biopsies from omental and subcutaneous AT in *paper I-IV* were immediately placed in RNAlater (Ambion,) and then frozen at – 80ºC according to the manufacturer's instructions.

Frozen adipose tissue was homogenized and total RNA extracted using the RNeasy Mini Kit (QIAGEN) according to the supplier's instructions including a DNase digestion step (RNase-Free DNase set, QIAGEN) to remove any contaminating genomic DNA. An Agilent 2100 Bio analyzer (Agilent Technologies) was used to confirm the quality of extracted RNA. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used to analyse the concentration of RNA. Three hundred ng of RNA from each sample was transcribed to complementary DNA (cDNA) by Invitrogen superscript first strand synthesis system for real time polymerase chain reaction (Invitrogen) or by Applied Biosystems cDNA-kit, using random primers. RNA and cDNA were stored at -80°C.

Gene expression studies

House keeping genes

A house keeping gene is encoding for a protein that is not affected of the variable studied, in this case stable during acute inflammation. House keeping genes are needed to compensate for minor differences in RNA concentration and in differed efficiency in the cDNA synthesis between the samples. To investigate which house keeping gene to use in the vaccination study group, cDNA from PBMCs from four of the subjects was analysed before and four hrs after vaccination using TaqMan Human Endogenous Control Plate (Applied Biosystems). In the open heart surgery study group cDNA from omental AT from four subjects was analysed before and after surgery using TaqMan Human Endogenous Control Plate (Applied Biosystems).

As Cyclophylin A demonstrated stability during inflammation and a similar cycle threshold value (Ct-value) to many of the genes of interest, it was used for the relative quantification (RQ) analyses in both study groups.

RT-PCR

To analyze gene expression of AT biopsies and PBMCs in *paper I and III* 3 µl of cDNA were mixed with TaqMan Universal PCR master Mix (2x) (Applied Biosystems) and primer-probe mix (20x) with TaqMan Gene Expression Assays (Applied Biosystems) to a final volume of

25µl and all samples were run in triplicates. The gene expression assays used for quantitative RT-PCR (TaqMan®) are all described in detail in each paper.
To analyze AT gene expression in study *II-III*, cDNA was mixed with TaqMan® Universal PCR master Mix (2x) (Applied Biosystems) in a total volume of 90 µl and all samples were run in duplicates. The samples were loaded onto a TaqMan Low Density Assay plate (Applied Biosystems). Primers for the target and hose keeping gene expression assays (Applied Biosystems) are listed in detail in each paper.

Immunohistochemical staining of adipose tissue sections

The gene expression results were confirmed by immunohistochemistry in *paper II and III*. Immunohistochemical staining was performed on biopsies from omental and subcutaneous AT to investigate active NF-κB-p65, the protein of E-selectin (anti-CD62E) and presence of macrophages (CD68) in *paper II* and to investigate staining intensity and localization of PAI-1 antigen in *paper III*.
Staining was performed using a standard protocol on serial sections from 4-5 µm thick formalin-fixed paraffin-embedded sections. Following antigen retrieval procedure using heat the sections were incubated with primary antibodies, as described in detail in each paper. Phosphate buffer saline was used in all subsequent washes. Positive immune reactivity was visualized using Avidin-Biotin peroxidase Complex (Vector Laboratories) and developed using a DAB-kit (Vector Laboratories) according to the instructions of the manufacturer. Sections were counterstained with haematoxylin.
Evaluation of the immunohistochemical staining included only subcutaneous AT where the number of positive cells was estimated in relation to the number of vessels in every paired biopsy. A semi-quantitative scale from 0 to +++ (where 0 is no positive cells, + is < 25% positive cells, ++ is 25-75% positive cells and +++ is > 75% positive cells) was used. The evaluation was performed blindly by two independent investigators. The agreement between the different investigators was > 90 %. The discrepancy was never more than one scale step and consensus was obtained by re-evaluation.

Microdialysis

Microdialysis is a validated method to gain access to and sample tissue derived molecules from the intercellular space. In brief, the basic principle is to copy the function of a capillary blood vessel by perfusing a dialysis membrane, inserted in the tissue, with a physiological liquid. The dialysate could then be analyzed and reflects the composition of the extracellular fluid due to the diffusion of substances back and forth over the membrane. Using microdialysis enables a continuous sampling for hours to days without withdrawing any blood [123-125], see Figure 7. In the studies by Dostolova and Murdolo there have been indicated a risk of an artefact due to the trauma from the catheter itself. Possibly, this risk may depend on which insertion technique and catheter membrane that was used. To avoid this artefact risk we used a two catheter membrane protocol where the second membrane was inserted two and a half hrs after the first membrane, assuming that local catheter-induced inflammation would result in similar IL-6 dynamics in both catheters.
Local production of IL-6 was studied by microdialysis in subcutaneous AT during open heart surgery in four patients, representative for the whole study group regarding age, BMI and the CPB-time. At start of surgery a microdialysis catheter (CMA 71, CMA Microdialysis) with a 100 000 Da cut-off and a membrane length of 30 mm was inserted subcutaneously under sterile conditions in the upper left quadrant of the abdomen without using local anaesthetics.

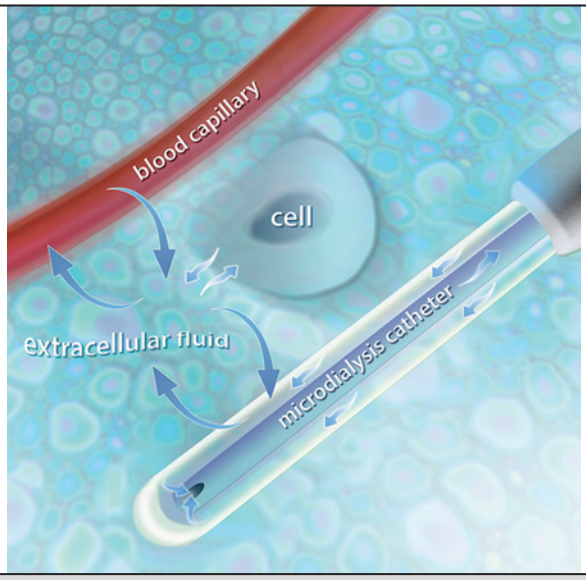

Figure 7. The basic principle of microdialysis is to mimic the capillary blood vessel. A dialysis membrane is inserted in the tissue and perfused with a physiological liquid. The dialysate could be analyzed, and reflects the composition of the extracellular fluid due to the diffusion of substances back and forth over the membrane. Using microdialysis enables a continuous sampling for hours to days without withdrawing any blood.
Reprinted by permission from CMA Microdialysis AB.

The catheter membrane was inserted guided by a splitable introducer (CMA Microdialysis) where after the introducer immediately was drawn back and the catheter membrane was left uncovered in the tissue. The catheter was covered with a sterile dressing membrane and immediately connected to a microdialysis pump (CMA 107, CMA Microdialysis) and infused with Ringer-acetat (Fresenius Kabi) with a flow-rate of 1 µl/min. The dialysate was collected at 30 min intervals for 330 min. Collected dialysate up to 60 min after catheter insertion was not included in the statistical analysis because of the normal equilibrating and fluid filling time period of one hour of the catheter syst [125]. The dialysate was collected into microvials (CMA Microdialysis) and immediately placed on ice and stored at -80º C.

Dialysate analysis

Dialysate was analysed for lactate concentrations using a photometric method (ISCUS, CMA Microdialysis) and for IL-6 levels using ELISA (Human IL-6 DuoSet, R&D Systems). Intra- and inter-assay coefficients of variations were 9.4% and 15.0%, respectively.

Statistical analyses

Data are presented as median (interquartile range), median (min-max), mean ± Standard Error of Mean (SEM) or numbers (percent). Differences between groups were compared using Mann-Whitney U-test (skewed data), Wilcoxon matched-pairs signed-rank test or student's t-test. A test for linear trend was used to evaluate the differences in plasma levels of PAI-1 over time after surgery, in *paper III*.

To minimize the risk of a type I error due to the limited number of study subjects and multiple testing in paper II, only gene expression results were considered significant for genes where all samples showed an increased relative quantification after surgery.
A mixed linear model was used to evaluate the effect of microdialysis catheters over time on IL-6 in dialysate, in *paper II*. Time was entered in the model as a categorical variable with four different time points and an interaction term was included in the model to test for heterogeneous differences between catheters. Because of skewed data and an increase of variation with time log transformed IL-6 was used in the final analysis. There is always a risk of finding at least one significant difference when doing multiple testing even if the overall null hypothesis that none of the variables differ between groups is true. The Bonferroni procedure is sometimes used to correct for this. One problem with this method is that it is conservative in the usual multivariate situation when the comparisons are not independent. In our study with dependent variables it is not desirable to control the error rate of the whole study, since this would reduce the power and therefore may miss relevant and significant differences.
The level of significance was specified at <0.05

RESULTS AND DISCUSSION

Paper I

We investigated if a standardised inflammatory stimulus could activate AT inflammation and circulating PBMCs, using vaccination against Salmonella typhi as a model of inflammation. Since current knowledge about stimulated inflammatory activity in different cell types and tissues is mostly based on animal and *in vitro* studies, it is of particular interest to investigate this in humans. Eighteen healthy volunteers were included in the study. Every second subject was allocated to vaccine or control group. Gene expression of IL-1β, IL-6 and TNF were investigated in AT and PBMCs. Relative quantification of gene expression was calculated with cyclophylin A as a house keeping gene.

Systemic inflammatory markers

No differences were found between the two groups regarding basic characteristics (Table, *paper I)*. We found higher plasma levels of IL-6 in the vaccine group 8 hrs after vaccination, 4.86±9.3 pg/ml compared to 1.6±2.0 pg/ml in the control group (Figure 2, *paper I).*

Gene expression of inflammatory markers

Our results demonstrated an increased gene expression of TNF in PBMCs 4 hrs after vaccination when compared to the control group, but no differences were found in gene expression of IL1-β and IL-6 between the two groups, Figure 8a.

In AT there were no differences in mRNA gene expression of IL1-β, IL-6 or TNF between the vaccine and the control group, Figure 8b. The gene expression of IL-6 was higher in AT compared to PBMCs and the gene expression of IL-1β was higher in PBMCs compared to AT taking vaccinated and controls together. In this context it's noteworthy that the mRNA expression of the house keeping gene was similar in both AT biopsies and PBMCs.

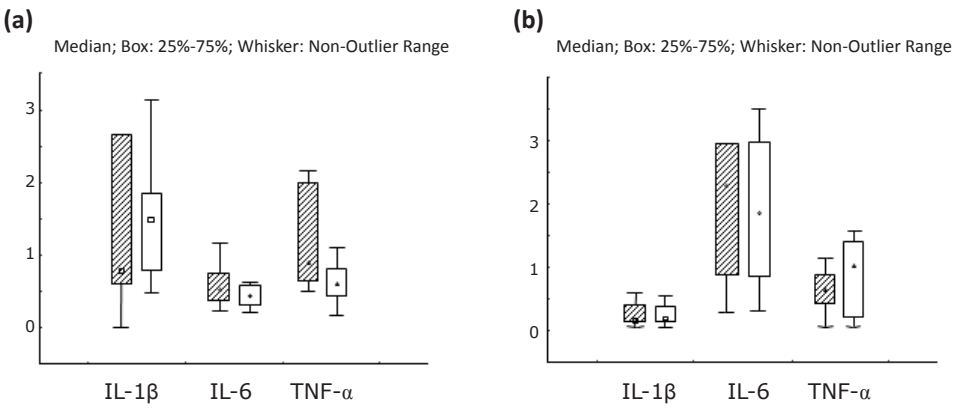

Figure 8. RNA expression of inflammatory cytokines in **(a)** peripheral blood mononuclear cells (PBMC) and **(b)** adipose tissue. Indexes of quantitative RNA gene expression of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) in relation to the house keeping gene cyclophylin A. Vaccinated subjects in striped boxes and controls in open boxes.

Discussion

The higher plasma levels of IL-6 in the vaccination group support the experimental model of systemic inflammation. An increased IL-6 gene expression in PBMCs could however not be found 4 hrs after vaccination, perhaps due to kinetics or that the IL-6 producing monocytes mainly are differentiating into macrophages and trapped in the tissue at the site of vaccination. We found a four-fold increase of circulating IL-6 following vaccination. Previous results from our group have shown ten-fold increases after vaccination [126]. However, compared to the previous study, our subjects did not have an indwelling peripheral vein catheter during the study which probably could explain the difference in peak plasma IL-6 levels; otherwise the study protocol was similar. Genetic variation is an important factor with possible impact on circulating levels of IL-6. However, there have been conflicting data whether genetic variation in the IL-6 gene could explain variability in stimulated plasma levels of IL-6 [119, 127-129]. Results from our group have clearly demonstrated that the G-allele of the -174 G>C polymorphism in the promoter region of the IL-6 gene is associated with increased IL-6 levels in plasma after vaccination [126]. Trying to control for genetic differences, all subjects were homozygous for the common -174 G allele.

Although vaccination is not believed to result in increased plasma TNF levels [101, 126], we found an increased gene expression of TNF in PBMCs 4 hrs after vaccination when compared to the control group but no differences regarding gene expression of IL1-β and IL-6 between the two groups. The reason for these apparently divergent findings is not fully understood but a possible explanation is that the increased TNF gene expression in PBMCs found in the present study derives from a subset of activated monocytes/macrophages released from the vaccinated tissue. This increase of TNF gene expression might not be substantial enough to result in increased plasma levels of the protein. However, according to our results TNF is a more relevant stimulus than IL-1β in stimulating IL-6 thereby extending previous studies in animals and *in vitro* [68].

Human subcutaneous AT can release IL-6 *in vivo* [41]. Our results are in agreement with this finding by demonstrating unstimulated IL-6 gene expression in AT. Interestingly, the basal level of mRNA gene expression was four-fold higher in AT compared to PBMCs. However, we were not able to stimulate AT by vaccination. In contrast, there seems to be an increased transcription of IL-6 mRNA in human adipocytes following *ex vivo* LPS stimulation [130]. Furthermore, patients with chronic inflammation have increased IL-6 gene expression in AT compared with patients with normal levels of inflammatory markers [45], suggesting that AT actually responds to an inflammatory stimulation. Further *in vivo* studies with stronger stimulus to inflammation are needed to resolve this issue.

We chose to collect AT biopsies only once, at 4 hrs and we cannot exclude that AT biopsies at other time-points would have generated a different result. The time point was chosen because of experiences from an earlier study in our group that had shown increasing circulating IL-6, 4 hrs after vaccination [126]. The reason for taking biopsies only once was that we wished to avoid the risk of an uncontrolled systemic inflammation due to the local trauma caused by the biopsy. This step of precaution led us to compare vaccinated subjects with non-vaccinated instead of using subjects as their own controls which would have reduced the risk of inter-individual variation to a minimum. Although the biopsy did not seem to stimulate systemic inflammation in our study, a recent report demonstrated increased local inflammation up to 4 hrs after an open AT biopsy [131] why this step of precaution may have been justified after all.

In summary, vaccination activated systemic inflammation with increased TNF-α gene expression in PBMCs but did not trigger proinflammatory gene expression in AT. Also, we found a higher basal gene expression level of IL-6 in AT than in PBMCs. Further *in vivo* studies including a stronger stimulus to inflammation are needed to elucidate the inflammatory capacity of AT.

Paper II

Since vaccination was too weak to provoke AT inflammation we examined patients that were planned for open heart surgery with CPB, which is a stronger *in vivo* model of induced acute inflammation in humans, to further elucidate the inflammatory capacity of AT.

Acute systemic inflammation

Basic characteristics of subjects are described in detail in Table 1, *paper II*. After approximately 30-40 min of surgery and before institution of CPB, blood samples and the first biopsies from omental and subcutaneous AT were collected. At the end of surgery, when the patient had been weaned off CPB, a second sampling of blood and AT biopsies was collected.
Open heart surgery with CPB induced a 25-30 fold increase in plasma IL-6 levels 3.0 pg/ml (1.9-5.9) before and 79.8 pg/ml (31.5-190.8) after CPB, Figure 9a.

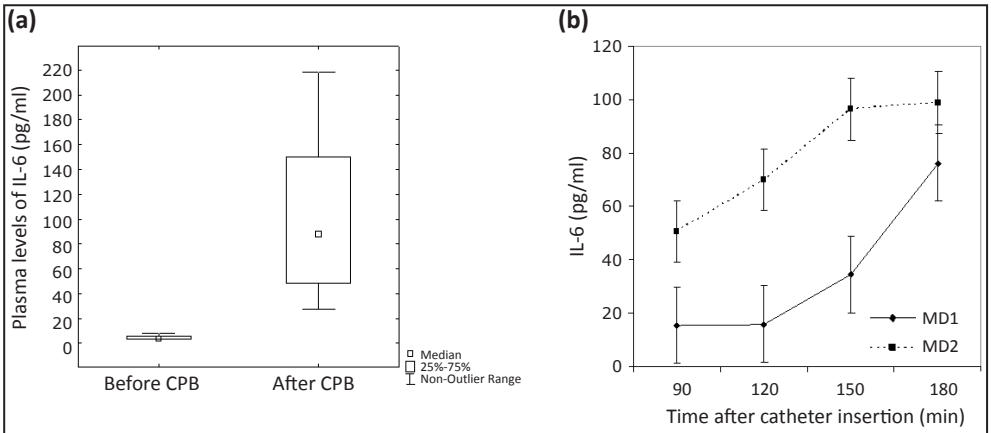

Figure 9. **(a)** Plasma levels of IL-6 before and after open heart surgery (p=0.008, n=14). **(b)** IL-6 levels in microdialysate from subcutaneous AT during and after open heart surgery focused on the time period from 90 to 180 min after catheter insertion with both catheters in parallel. Dialysate from the 1st microdialysis catheter (MD1, n=4) showed increased production of IL-6 ~ 3 hrs after start of surgery, and the 2nd microdialysis catheter (MD2, n=3), inserted 2.5 h after MD1 demonstrated increased IL-6 production already after 90 min with 2.3x higher mean levels of IL-6 vs. MD1 (p=0.005).

Gene expression of proinflammatory markers in omental and subcutaneous adipose tissue

Relative quantification of adipose tissue mRNA gene expression after open heart surgery was calculated in relation to the house keeping gene cyclophylin A with the first biopsy in every paired sample as a reference, and expressed as change from baseline. IL-6, IL-8/CXCL8 and E-selectin showed a 100-fold increase in mRNA expression in omental AT and

in subcutaneous AT these genes increased even more, up to 200-400-fold. The pattern of increased proinflammatory chemokine and cytokine gene expression was similar in omental and subcutaneous AT, Figure 10a-b.
The mRNA expression of receptors and transcription factors in omental and subcutaneous AT are presented in Figure 10c-d. In omental AT there was increased mRNA expression of IL-1R1, NF-κB1, TLR2, TLR4 and TNFR-SF1A. A similar pattern was found in subcutaneous AT with increased mRNA expression of FOS, JUN, TLR2 and TNFR-SF1B.

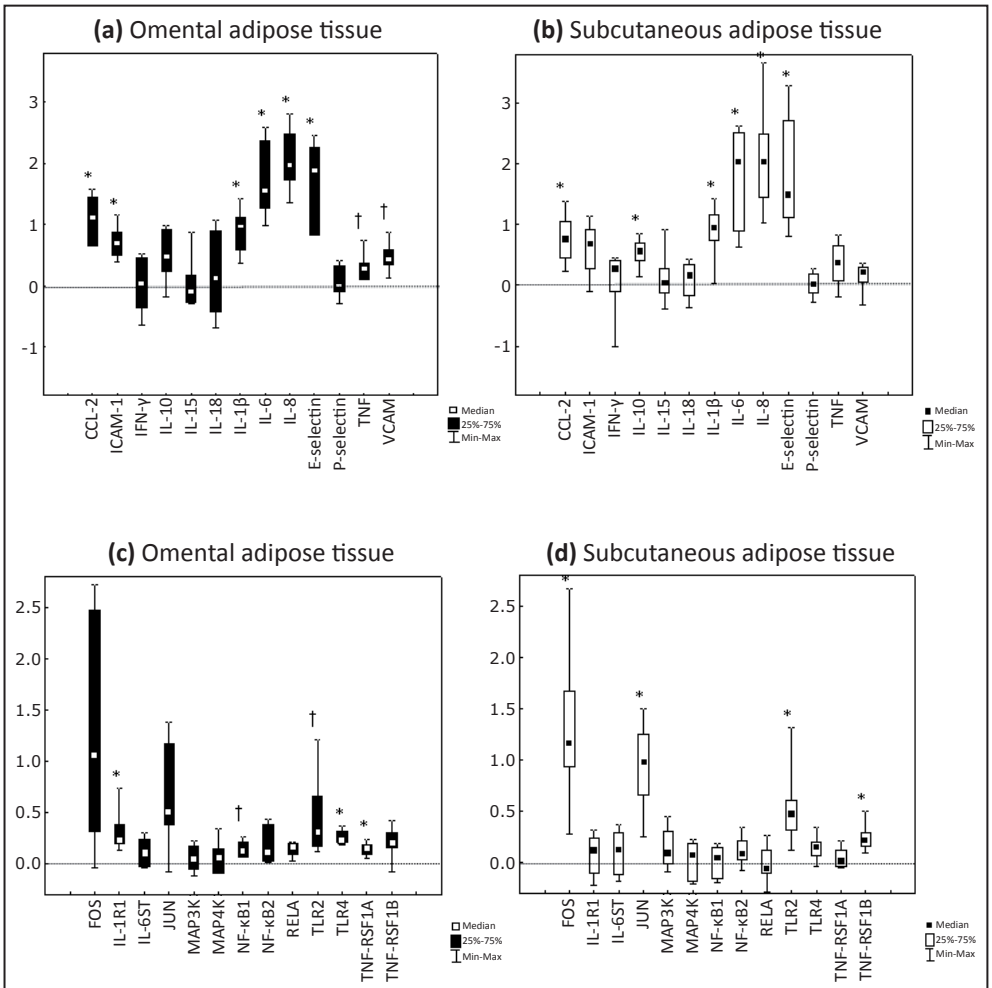

Figure 10. Relative adipose tissue mRNA gene expression after open heart surgery expressed as change from baseline.
Relative quantification (RQ) of mRNA gene expression of chemokines and cytokines **(a and b)** and receptors and transcription factors **(c and d)**. RQ levels expressed in relation to the house keeping gene Cyclophylin A, using the first biopsy in every paired analysis as a reference and a logarithmic scale. Omental adipose tissue (7 patients) and subcutaneous adipose tissue (8 patients). * $p<0.05$, † $p<0.02$

Gene expression findings verified by immunohistochemistry staining

To verify the increased mRNA expression on a protein level, staining and localization of active NF-κB-p65 was investigated by immunohistochemistry in AT biopsies. There was a marked staining of NF-κB-p65 in subcutaneous AT following surgery. NF-κB-p65 was localized to adipocyte nuclei, the endothelium and macrophages as illustrated in Figure 11 a-b. Moreover, E-selectin stained strongly in the vascular endothelium after surgery, Figure 11 c-d. Finally, we found a high number of macrophages in close contact with and in the vascular wall, Figure 11 e-f. Immunohistochemistry staining of omental AT biopsies showed a similar result as for subcutaneous biopsies but with less intensity.

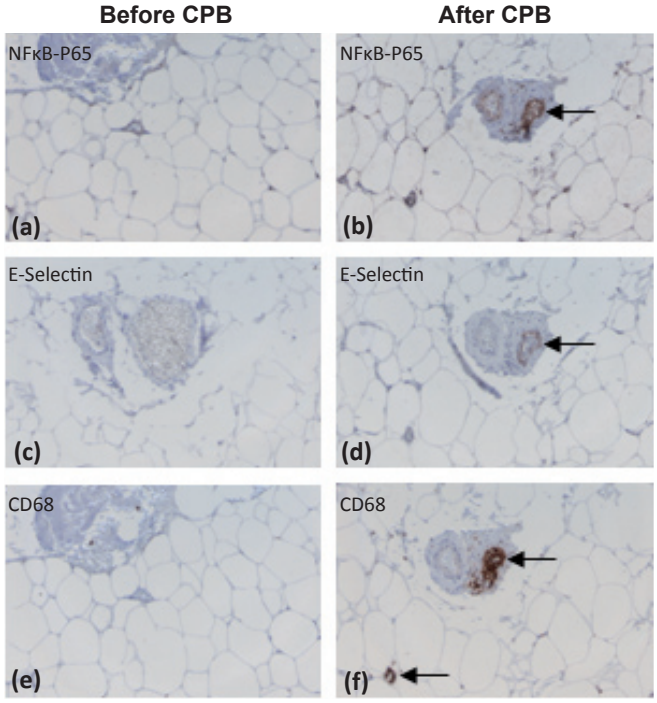

Figure 11. Representative micrographs of serial sections of subcutaneous adipose tissue before and after open heart surgery with cardiopulmonary bypass (CPB) demonstrating a marked upregulation of NF-κB-p65 **(a and b)** and a strong staining of E-selectin in the vascular endothelium **(c and d)** after CPB. There were also a high number of CD68 positive cells in close contact with the vascular endothelium migrating into the vessel wall (e and f). Arrows mark areas with strong positive staining.

IL-6 in microdialysate from subcutaneous adipose tissue

Local production of IL-6 in subcutaneous AT was analysed using microdialysis during and after open heart surgery. In total, IL-6 was measured in 7 microdialysis catheters; Dialysate was collected every 30 min and IL-6 increased in the dialysate from 14 (5-25) pg/ml, at 90 min to a level of 147 (49-358) pg/ml, at 5 hrs ($p<0.05$). To avoid artefacts and to discriminate between local inflammation and secondary activation of AT inflammation by surgery, we used a second catheter membrane inserted at the end of surgery, two and a half hrs after the

first membrane, assuming that local catheter-induced inflammation would result in similar IL-6 dynamics in both catheters. In dialysate from the first microdialysis catheter (n=4) there was an increased production of IL-6 approximately 3 hrs after start of surgery whereas in dialysate from the second microdialysis catheter, inserted 2.5 hrs after the first catheter, an increased IL-6 production was detected already 90 min after insertion (n=3). The mean levels of IL-6 in the second catheter were 2.3 times higher than the levels in the first catheter, Figure 9b. During this period the lactate levels in dialysate were stable and did not increase in any of the patients. The lack of changes in lactate levels confirms a robust and well functioning microdialysis system.

Discussion

Although several studies have demonstrated an inflammatory capacity of AT [37, 41, 44-46, 50-54, 124, 130, 132-135], the mechanism that triggers AT inflammation is still unknown. The present study not only confirms the results of these studies but also extends the knowledge about AT inflammatory capacity, by adding data from omental AT. Furthermore, whereas previous studies lack information about stimulated AT inflammation on a cellular level, our immunohistochemistry results clearly demonstrated that macrophages and vascular endothelium (E-selectin) of AT blood vessels became activated by open heart surgery. In addition, our findings from the microdialysis experiment confirm the results from the gene expression experiment and may indicate that the NF-κB-regulatory pathway is the key through which IL-6 synthesis in adipocytes is regulated. However, a contribution of IL-6 from macrophages and endothelium in AT blood vessels cannot be excluded. The AT IL-6 content after operative trauma has earlier been found to correlate with circulating levels of IL-6 [132] but in our study, we found increased gene expression followed by IL-6 production in subcutaneous AT, measured by microdialysis, making it less likely that IL-6 in AT merely reflects plasma levels.

The results of the present study are consistent with earlier studies that demonstrated an extensive acute systemic inflammation with high plasma levels of both IL-6 and TNF following open heart surgery [111, 136]. The acute phase response following CPB has been associated to increased gene expression of inflammatory mediators in circulating leukocytes, possibly due to contact of circulating blood with the synthetic surface of the CPB system [113, 114].

We speculated that circulating TNF activates the vascular endothelium and the surrounding AT to produce E-selectin and other inflammatory mediators through the NF-κB signalling pathway. Secondary to the expression of E-selectin and adhesion molecules on the vascular endothelium, circulating monocytes are recruited and activated to differentiate into macrophages with the possibility to extravasate into the surrounding AT. This could be one explanation for the recruitment of macrophages to AT resulting in the low-grade chronic inflammation seen in obese subjects [47]. Another explanation for recruitment of macrophages into AT in obese individuals is increased levels of free fatty acids that can induce NF-κB activation by TLR4 followed by expression of cytokines, some of which suppress insulin signalling [13]. However, the mechanisms for triggering AT inflammation could differ in low-grade chronic and acute systemic inflammatory conditions.

It is well documented that the injury inflicted by surgery evokes increased release of cytokines and stress hormones [137]. However, these are not solely produced in AT, other sources such as skeletal muscle have also been demonstrated with increased mRNA expression

of proinflammatory markers after surgery [138]. A central effect of these mediators is the development of insulin resistance [137], supported in previous studies which have reported on an improved glucose tolerance after neutralization of TNF in obese rodents [139, 140].
Our results give further support to an AT inflammatory capacity in contribution to the acute systemic inflammation and thereby possibly aggravating the increased insulin resistance seen during surgery [137]. In these studies, this effect was transient and therefore may be of minor long-term clinical relevance, but if AT recruitment of macrophages during the acute phase of inflammation also contributes to the low-grade chronic AT inflammation seen in obese individuals, it may have a deeper impact on metabolic disorders and long-term clinical relevance. Indeed, we found a positive correlation between the need for insulin during the first 24 hrs after start of surgery and relative quantification of IL-1β gene expression (R=0.86, p=0.014) and non-significant positive trends between the need for insulin and relative quantification of CCL-2 and IL-6 gene expression in omental AT after surgery. However, due to the low number of study subjects it is impossible to draw any definitive conclusion from the correlation analyses. The need for insulin together with activation of AT inflammation could be investigated in a future study of the role of obesity during surgery.

In summary, systemic inflammation stimulates AT to produce an innate inflammatory response due to upregulation of the NF-κB regulatory pathway. First, gene expression of several proinflammatory genes increased dramatically after open heart surgery with a similar pattern in both omental and subcutaneous AT. Second, the increased gene expression was confirmed by immunohistochemistry showing strong expression of E-selectin with a high number of macrophages in close contact with and in the vascular wall. Third, increased gene expression of inflammatory mediators was followed by increased production of IL-6 in subcutaneous adipose tissue measured by microdialysis. It can be hypothesized that AT exerts a modulatory effect on innate immunity in humans and that the inflammatory response aggravates insulin resistance.

Paper III

CVD events such as myocardial infarctions are known complications after surgery and severe infection where acute systemic inflammation is a common denominator. In the light of our previous findings on AT inflammation we proceeded with studies on plasminogen activator inhibitor-1 (PAI-1) synthesis in AT after acute systemic inflammation, in order to investigate a possible mechanism behind these complications.

Plasma levels of IL-6 and PAI-1 during and after open heart surgery

To investigate circulating concentrations of IL-6 and PAI-1 antigen, blood samples were collected before and after open heart surgery with a median time of 125 (90-285) min between the paired samples. We confirmed our findings from *paper II*, with the observation of a more than 25-fold increase in plasma IL-6 levels, in median 3.1 pg/ml (3.0-5.9) before and 85.2 pg/ml (31.5-218.2) after surgery.
No significant differences were found in plasma levels of PAI-1 antigen analyzed before and after CPB (Figure 3a *Paper III)*. In subjects further investigated with plasma samples every hour up to 6 hrs after start of surgery, a positive linear trend was found in plasma levels of PAI-1 antigen with an increase after 4 hrs after start of surgery (Figure 3b *Paper III)*.

PAI-1 gene expression in adipose tissue

There was an increased mRNA expression of PAI-1 in both omental and subcutaneous AT after open heart surgery, Figure 12. When these results are expressed as relative quantification using the first biopsy in every paired sample as a reference, the gene expression of PAI-1 in omental AT showed a 27-fold increase after surgery whereas a 3-fold increased gene expression was found in subcutaneous AT. The degree of PAI-1 mRNA increase was higher in omental compared to subcutaneous AT. No difference was found in basal PAI-1 gene expression between the two AT depots.

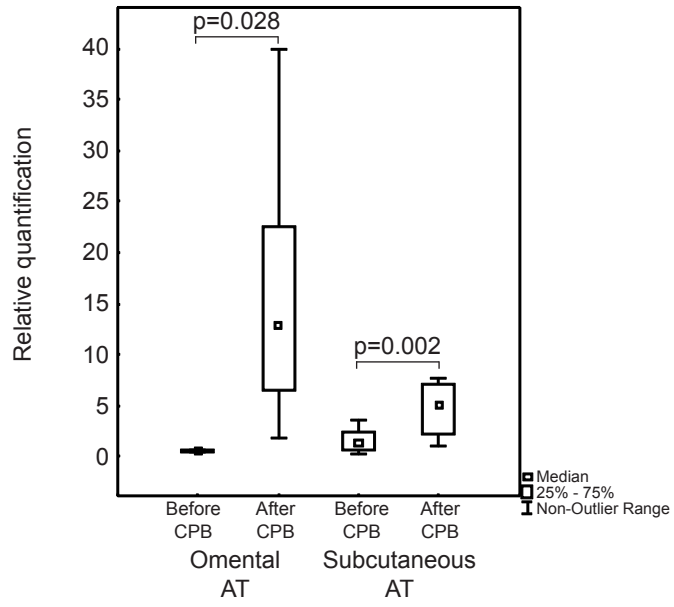

Figure 12. Relative quantification of plasminogen activator inhibitor-1 (PAI-1) gene expression in omental and subcutaneous adipose tissue (AT) before and after open heart surgery with cardiopulmonary bypass (CPB). Gene expression of PAI-1 expressed in relation to the house keeping gene cyclophylin A. No difference was found in basal levels of PAI-1 gene expression between the two different AT depots. After open heart surgery there was increased mRNA expression of PAI-1 in both omental (p=0.028, n=7) and subcutaneous AT (p=0.002, n=12). Median time between samples was 125 (90-285) min.

Staining for PAI-1 protein in adipose tissue

To confirm production of PAI-1 at a protein level and to investigate the localization of PAI-1 in AT, we stained for PAI-1 antigen in subcutaneous AT biopsies. There was a marked increased staining of PAI-1 antigen detected within the vascular endothelium as well as in the adventitia of AT vessels after open heart surgery. PAI-1 antigen was also detected in the AT interstitium close to AT vessels and in solitary cells in between adipocytes, Figure 13.

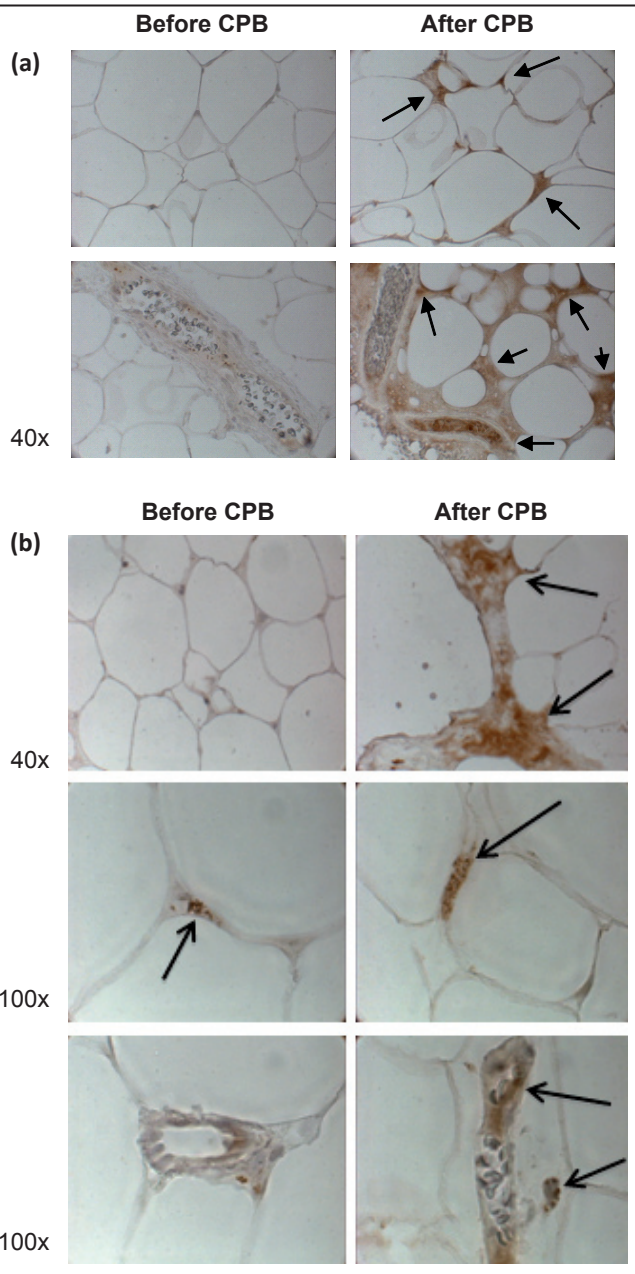

Figure 13. Localization of plasminogen activator inhibitor-1 (PAI-1) antigen in subcutaneous and omental adipose tissue (AT) before and after open heart surgery.
Representative immunohistochemical analyses of paraffin sections showing vasculature and adipocytes in subcutaneous **(a)** (n=5) and omental **(b)** (n=1) AT before and after cardiopulmonary bypass (CPB). Strong staining of PAI-1 was detected within the endothelial cells as well as in the adventitia, after surgery. PAI-1 antigen was also detected in the AT interstitium close to AT vessels and in solitary cell nuclei in between adipocytes.

Discussion

Our results, with a markedly higher PAI-1 gene expression in omental compared to subcutaneous AT following surgery, underlines the importance of abdominal fat distribution in obesity. An intensified synthesis of PAI-1 in human AT following stimulation could severely impair the fibrinolytic activity in plasma and may therefore explain the risk of myocardial infarction seen after surgery or infection. The PAI-1 response in omental AT following stimulation could also be one of the explanations why obesity is an independent risk factor for myocardial infarction [12].

Our gene expression results confirm earlier *ex vivo* studies where higher PAI-1 synthesis in omental versus subcutaneous cultured adipocytes following stimulation was demonstrated [91, 141]. Another study showed a lower *ex vivo* secretion rate of PAI-1 in omental compared to subcutaneous AT [142], however, this study investigated PAI-1 production in non-stimulated conditions. Other studies have not been able to demonstrate any differences between the two different AT depots. Basal arterio-venous concentrations of PAI-1 antigen across omental and subcutaneous AT were measured, but neither of them could show any significant differences across different AT beds *in vivo* [81, 143]. Nevertheless, the contribution of different AT beds to circulating PAI-1 could differ in health and disease. A greater arterio-venous difference in PAI-1 activity in diabetic compared to non-diabetic subjects has been reported, but the groups were small and the results were not paralleled by PAI-1 antigen concentrations.

Ex vivo experiments have shown that angiotensin II stimulates PAI-1 gene expression in human adipocytes, a stimulation which was completely blocked by angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ACEi/ARBs) [99]. The use of angiotensin II inhibitors has also been reported to alter fibrinolytic activity during open heart surgery [144]. In *paper III*, six out of twelve subjects were treated with ACEi/ARB medication, all with maximal doses. However, we could not find any differences in PAI-1 gene expression between patients with or without angiotensin II blocking medication. Hence, we conclude that angiotensin II is not a likely major stimulatory factor of PAI-1 synthesis in AT during acute systemic inflammation.

Notably, we found a markedly increased gene expression of PAI-1 and a strong staining of PAI-1 antigen in AT biopsies taken already when the patient had been weaned off CPB, while plasma levels of PAI-1 did not increase until hours after surgery. This clearly suggests that the PAI-1 protein detected in AT precedes the increase in plasma PAI-1 levels, a finding that could be highly relevant. It indicates that PAI-1 synthesis in AT significantly contributes to the later increase in plasma concentrations of PAI-1.

The results of the present study are of potential major clinical relevance. The increased risk of myocardial infarction seen after major surgery does not appear during the surgical procedure itself, characterized by the stress from anaesthesiology and surgical trauma, but occurs during the early postoperative period [18]. The same phenomenon is found after a severe infection with the highest risk of myocardial infarction during the first week after onset of infection [19]. Both surgery and infection result in an acute systemic inflammation and the delay in time indicates that putative risk factors for myocardial infarction are synthesized as a response to the acute phase reaction. Support for this hypothesis is that chronically elevated levels of PAI-1 are associated with spontaneous coronary arterial thrombosis in transgenic mice [84] and that increased activity of PAI-1 on the first day after CABG correlates to early vein graft occlusion [145]. Other systems important for coagulation and haemostasis may also react upon an acute systemic inflammation; increased plasma levels of activated factor VII following an acute inflammation [103] and platelet activation after bacterial infection [87, 146].

The merged results from *Paper II and Paper III* indicate that PAI-1 synthesis in AT is regulated

through the NF-κB signalling pathway, congruent with a recent review by Kruithof[147] and supported by previous findings from animal studies [94] and *ex vivo* experiments on human adipocytes [91] where LPS or TNF were used as inflammatory stimuli.

In summary, - PAI-1 gene expression and protein synthesis in AT was induced after acute systemic inflammation. The increase was most prominent in omental AT. PAI-1 produced by AT preceded the increase in plasma levels of PAI-1 and may be a link between inflammation and impaired fibrinolytic activity causing myocardial infarction.

Paper IV

Regulation of adipokines in the early phase of an acute inflammation in humans is of considerate interest as they are suggested to have an important role also in chronic inflammation and in the development of atherosclerosis. However, the control mechanisms of adiponectin and leptin during an acute inflammatory response are still poorly understood. *Paper IV* is a sub-study to the studies in *paper I and II* where acute inflammation was induced by vaccination and open heart surgery respectively, with the aim to investigate the effects on adiponectin and leptin synthesis.

Plasma levels of adipokines and IL-6

Plasma levels of adiponectin and leptin were unaltered after vaccination, showing similar levels as in the control group (Figure 1, *paper IV*) although the plasma levels of IL-6 increased 3-4-fold with a significant difference at 8 hours after vaccination.

Gene expression of adipokines

As open heart surgery with CPB is a strong inducer of systemic inflammation, we analyzed relative mRNA gene expression for adiponectin and leptin after open heart surgery, expressed as change from baseline in both omental and subcutaneous AT biopsies. Neither adiponectin nor leptin mRNA from omental AT did change after open heart surgery with a median time of 125 (90-285) min between the biopsies. Similar results were obtained for subcutaneous AT, Figure 14.

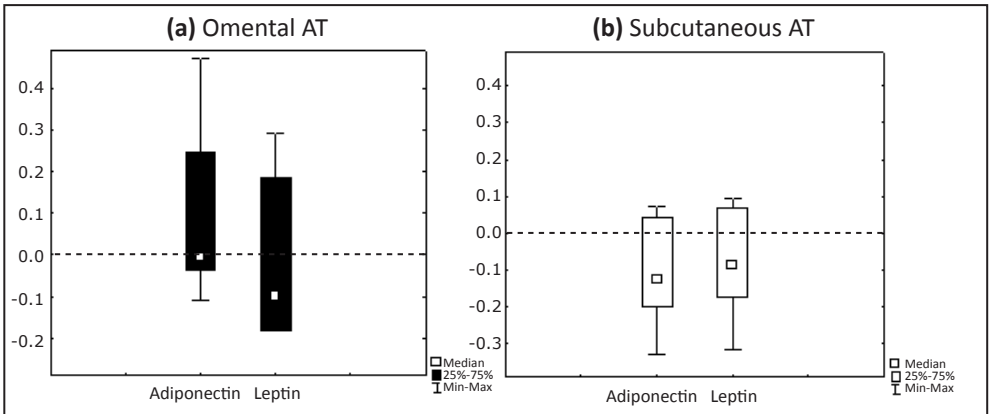

Figure 14. Relative AT mRNA gene expression of adiponectin and leptin **(a)** in omental AT and **(b)** subcutaneous AT after open heart surgery, expressed as change from baseline.
Relative quantification expressed in relation to the house keeping gene cyclophylin A, using the first biopsy in every paired analysis as a reference and a logarithmic scale. Omental AT (n=7) and subcutaneous AT (n=8).

Discussion

Recent studies regarding circulating concentrations of adiponectin and leptin during acute systemic inflammation has demonstrated a reduction of adiponectin, inversely correlated to C-reactive protein with a decrease 24 hrs after myocardial infarction [148]. Furthermore, leptin increase in plasma following open heart surgery, but not until 24 hrs post-surgery [149]. There are also some recent *in vivo* studies that have focused on both adiponectin and leptin synthesis in relation to acute inflammation, where leptin levels increased in plasma after LPS injection but adiponectin levels remained unchanged [52].

However, in our study we did not find any differences in plasma levels of adiponectin or leptin up to 24 hrs after vaccination. One explanation is that our vaccination model was too weak to induce a potent systemic inflammation, something which is supported by the very modest increase in IL-6 plasma levels (*Paper I*). Despite a strong systemic inflammation induced by open heart surgery, there was still a lack of changes on a gene expression level of adiponectin and leptin.

Possible reasons for this could be that that we have analyzed the AT gene expression too early or that these adipokines are not key elements in the acute-phase response. However, our results do not exclude that adiponectin and leptin levels change later during the inflammatory response.

Recent *in vivo* studies have focused on both adiponectin and leptin gene expression in relation to acute inflammation. Anderson and co-workers showed that LPS suppressed adiponectin gene expression in subcutaneous AT, whereas there was a trend towards increased gene expression of leptin [52]. Jernås and co-workers found no effect of inflammation, caused by subarachnoidal haemorrhage, on adiponectin and leptin mRNA gene expression in subcutaneous AT [150]. The reason for these somewhat discrepant results is not clear, but methodological issues have to be considered. First, the stimulus to systemic inflammation was different between the studies. Anderson *et al* [52] used LPS whereas we (in the open heart surgery study group) and Jernås *et al* [150] used tissue damage. These are strong inducers of systemic inflammation but might activate different subsets of cells, including macrophages in AT. Also the time-frames were different.

One limitation in our study may be the low number of patients investigated in the gene expression experiment. However, we used paired AT biopsies, which minimize a possible inter-individual variation. Furthermore, the size of the study group was large enough to demonstrate a very rapid onset of a marked innate immune response in AT following systemic inflammation induced by open heart surgery [112].

Both models of stimulated systemic inflammation used in this study activate inflammation through the nuclear factor-κB regulatory pathway [105, 112] and interestingly, none of the models were shown to have any influence on the synthesis of adiponectin or leptin. This suggests that the nuclear factor-κB signalling pathway is not involved in the regulation of these two adipokines in an acute-phase response. This is also supported by a recent study by Diez and co-workers who showed that leptin was not found to act as an acute phase reactant but more as a marker of nutritional status in patients with pneumonia [151].

When leptin synthesis has been studied in cultured human subcutaneous adipocytes it has been demonstrated that TNF attenuated leptin mRNA gene expression but in contrast, induced an increased protein release [152]. Furthermore, the proinflammatory cytokines IL-1β and TNF

both decreased leptin gene expression and protein production *in vitro*, but still IL-1β was found to elicit an early release of leptin to circulating plasma [153]. Interestingly, the results of these two *in vitro* studies suggest a pre-formed pool of leptin in human AT with a paracrine regulation to which IL-1β and TNF could be important key regulators. However, this pool has not yet been identified and it is not clear whether human adipocytes stimulated *in vitro* adequately represent the situation on a tissue level *in vivo*.

In summary, despite the use of two models of induced *in vivo* systemic inflammation we found no evidence of an early regulation of adiponectin and leptin synthesis, indicating that these two adipokines are not key elements in an acute systemic inflammation in humans. Our results extend previous studies by also analyzing gene expression in omental AT.

Concluding remarks and future perspectives

This thesis presents the novel finding that an acute systemic inflammation, stimulated by open heart surgery, induces a strong inflammatory response in both omental and subcutaneous AT including adhesion of macrophages to activated endothelium and release of IL-6 from AT interstitium. Moreover, the AT inflammation results in increased AT synthesis of PAI-1, an increase which is most prominent in omental compared to subcutaneous AT, and followed by a later increase in plasma levels. Our results are both new and of clinical relevance. As always, new findings lead to new questions. Several research areas focusing on acute inflammation need to be further elucidated.

The human innate immune system has developed during evolution as an acute-phase response to local or systemic infections. As AT has been demonstrated to be an organ capable of producing several proinflammatory active components, it may potentiate the acute phase response thus serving as a survival benefit, beyond the advantage of being an energy depot. However, the possible benefits of AT most likely become disadvantageous with increasing overweight and/or obesity, as this is intimately associated with obesity-induced insulin resistance and diabetes mellitus predisposing to atherosclerosis and subsequent CVD [10]. This is also supported by animal studies [154]. However, recent studies have reported on an "obesity paradox" where obese subjects seem to have increased risk of morbidity but not mortality compared with lean or underweight subjects but this remains to be further investigated [155-158].

When we interpret results from studies on AT inflammation and its clinical implications, we have to underline the importance of differentiate a low-grade chronic inflammation from an acute systemic inflammation. As current knowledge regarding AT inflammatory capacity, to a great extent relies on experiments and studies done in non-stimulated or chronic inflammatory conditions, it is important to add these data from human studies, using different models of induced acute systemic inflammation.
Our findings regarding the correlation between increased IL-1β gene expression in omental AT and the need for insulin to keep normal glucose levels during surgery indicate that AT inflammation contributes to insulin resistance during surgery. Further, we found positive tendencies between the need for insulin and increased gene expression of the inflammatory associated molecules CCL-2 and IL-6 in omental AT after surgery. The importance of inflammation in the development of insulin resistance is supported by a recent study

demonstrating that non-insulin resistant obese individuals lack the inflammatory response that characterizes the insulin resistant obese individuals [159]. Unfortunately, the limited number of patients also being only men, make it impossible to do any definitive conclusions regarding insulin resistance and AT inflammatory response.
Probably, all obese individuals are at risk of developing insulin resistance why it would be of interest to measure circulating levels of IL-6 and the need for insulin together with AT inflammation during the peri-operative as well as the post-operative period in a substantial number of female and male patients, to further investigate if the inflammatory response to open heart surgery is stronger in overweight patients due to secondary activation of AT. Another way to test this hypothesis would be the use of a single dose of LPS as a model of induced systemic inflammation.
We used microdialysis to investigate proteins on a tissue level and confirm the gene expression results but only IL-6 levels were analyzed. In a future project it would be appealing to collect dialysate for a longer period, even days. We used ELISA to analyze the dialysate but there are other techniques, such as multi-plex systems that enable analysis of a great number of different cytokines and chemokines in small sample volumes.

Today, we do not know if obese individuals have a stronger inflammatory response due to their high percent body fat, compared to normal-weight or lean individuals. This need to be further investigated. In addition, inter-individual differences in the acute phase response could also be explained by different genotypes. In the vaccination study group we tried to control for differences in inflammatory response due to genotype by including only subjects homozygous for the common -174 G allele in the -174 G>C polymorphism which has been shown to be associated with increased plasma levels of IL-6 after vaccination [126]. However, in the open heart surgery study group, it was not possible to take IL-6 genotype differences into consideration. Instead we collected and analyzed paired tissue and blood samples which minimize the influence of inter-individual variability, thereby indirectly controlling for other genetic variations as well.

Another interesting clinical aspect is that body mass index is a risk factor for post-operative atrial fibrillation, and lipid-lowering therapy with statins may reduce this risk due to the anti-inflammatory properties of these drugs. Our data regarding the role of AT in the inflammatory response to open heart surgery could result in treatment alternatives in patients with overweight by considering statins for all patients in this category [160, 161].

Beyond diabetes mellitus, hypertension, and hyperlipidaemia, the close association between elevated plasma levels of PAI-1 and abdominal fat distribution has led to the inclusion of impaired fibrinolysis in an expanded definition of the metabolic syndrome [86, 93, 162]. Numerous studies have demonstrated that patients with the metabolic syndrome are at high risk for developing cardiovascular disease, venous thrombosis and pulmonary embolism [10, 163, 164]. Increased PAI-1 activity predict cardiovascular events in patients with a history of an earlier myocardial infarction [83] and elevated PAI-I levels are associated to spontaneous coronary artery thrombosis in mice [84]. In this thesis we showed that an acute systemic inflammation in humans activated AT inflammation through the NF-κB signalling pathway together with an increased gene expression and protein synthesis of PAI-1. Noteworthy, we found a similar pattern regarding genes encoding pro-inflammatory effects in both omental and subcutaneous AT and maybe, it is time to start considering AT as an organ/tissue of innate

immunity. Moreover, our results indicate that during inflammation and/or infection, it is the abdominal fat that plays the most important role for the synthesis of PAI-1 as well as for the development of insulin resistance. However, other sources of increased PAI-1 synthesis, such as activated endothelium and/or platelets also need further investigation.

PAI-1 synthesis in AT due to acute systemic inflammation may be the link between inflammation and impaired fibrinolytic activity that might explain the increased risk of acute myocardial infarction seen after surgery or infection. A PAI-1 inhibiting antibody could be a new therapeutic strategy trying to reduce this risk.

CONCLUSIONS

I. Vaccination activates systemic inflammation but does not trigger proinflammatory gene expression in AT.

II. Acute systemic inflammation induced a strong inflammatory response in both omental and subcutaneous AT, including adhesion of macrophages to an activated endothelium and release of IL-6 from AT interstitium.

III. Acute systemic inflammation increased gene expression and protein synthesis of PAI-1 in human AT and that this increase was more prominent in omental compared to subcutaneous AT.

IV. Despite the use of two models of stimulated systemic inflammation we found no evidence of an early regulation of adiponectin and leptin synthesis, indicating that these adipokines are not key elements in an acute systemic inflammation in humans.

ACKNOWLEDGEMENTS

This work has been performed at the Department of Medicine, Unit of Cardiology and during these years I have had the pleasure to meet and work side-by-side with students and experienced scientists. I have also had the advantage to participate in the educational programme for PhD students in Molecular Medicine at the Karolinska Institutet. I would like to thank all colleagues, both former and present and in particular I would like to acknowledge:

Per Tornvall, my principal supervisor. Thanks for your endless enthusiasm and optimism, your patience and for sharing your out-standing knowledge in clinical matters as well as in molecular medicine. Thank you for giving me the opportunity to work in your group – it has really been a pleasure.

Per Eriksson, my co-supervisor, thank's for all your help and advice during these years. Your knowledge in molecular medicine is impressive and you always find rationale solutions in difficult situations.

Cecilia Linde, Head of the Department of Cardiology, for believing in me and for providing an excellent atmosphere for the combination of daily clinic and research.

John Pernow, thank you for the positive and friendly atmosphere at the unit of Cardiology and for always encouraging scientific work and discussions.

Anders Hamsten, for providing an excellent environment for science at the Atherosclerosis Research Unit, Centre for Molecular Medicine.

Patients and volunteers, your contribution to science is important world-wide.

My co-authors:
Martin Halle, Staffan Bjessmo, Jan Liska, Maria Kolak, Rachel Fisher, Eva Sverremark-Ekström and Stefan Söderberg for a nice co-operation and for all good scientific advice.

Gunilla Forsell and **Claes Hofman-Bang**, my clinical mentors. Your skills in cardiology and internal medicine are impressive. What you don't know is probably not worth knowing and Claes, thanks for telling Per T about my interest in science.

Eva Wallgren, thanks for your excellent technical assistance in the open heart surgery studies and for your help with the layout of this thesis.

The other group members **Martin Halle** and **Sarah Jayne-Reilly** for your humour when sharing daily experiences from research. I hope for further co-operation in future projects.

Colleagues and friends at the Department of Cardiology at the Karolinska University Hospital, thanks for providing me time and support to fulfil this thesis. A special thank to my **room mates** for all non-scientific chats and coffee breaks.

Birgitta Grape, for your kindness and support when looking for patients to the open heart surgery studies.

Marita Wallin, for all help with ordering and for always knowing where things are.

Barbro Burt, for being such a great teacher in good laboratory practice

Sivonne Arvidsson, for always being helpful in the lab.

Camilla Hage, for kind assistance in the vaccination studies.

Camilla Lindell, for teaching me how to become a friend with the ISCUS-machine.

Jakob Bergström, for statistical analyses.

Magnus Mossfeldt, for helping me with computer problems.

Erik Sundström, for the valuable years during the educational program for PhD students in Molecular Medicine.

Everyone at the Department of Immunology at Stockholm University for showing such an interest in my research and for introducing me into the worlds of ELISA and immunohistochemistry.

Karin Peterson (CMA Microdialysis systems), for always being interested and helpful when we were about to start running the microdialysis experiments.

All my friends outside the work, for lots of fun and for your patience these years.

My father and mother in law, **Yngve** and **Britta** for all your kindness, enthusiasm and support during these years. In particular, thank you for always being there for Sofia and Edvin, also with very short notice, – all of us have always appreciated your presence.

My father and mother, **Lars** and **Lisbeth** for your love and support and for all time spent with my children playing “jaga”, making buns and much more.

My wife **Eva**, you are the most wonderful partner in life. Your love, humour and intelligence make life easy.

My lovely children **Sofia** and **Edvin,** thank you for your patience during these years and especially during the final work of this thesis. You are the most important persons in my life and make it worth living.

This thesis was supported by grants from the **Swedish Heart-Lung-foundation** and **Karolinska Institutet**.

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I

Letter to the Editor

Journal of INTERNAL MEDICINE

doi: 10.1111/j.1365-2796.2008.01998.x

Vaccination, a human model of inflammation, activates systemic inflammation but does not trigger proinflammatory gene expression in adipose tissue

Dear Sir,

Inflammation plays a key role in the development of atherosclerosis [1]. Many studies have emphasized inflammatory markers as prognostic for atherosclerosis and myocardial infarction [2, 3]. One of these, interleukin-6 (IL-6), is a multifunctional cytokine with an important role in host defence. It is involved in the development of cells and tissues as well as in different pathological conditions. IL-6 is not constitutively produced but can be synthesized in response to inflammatory stimuli such as interleukin-1β (IL-1β), lipopolysaccharide and tumour necrosis factor-alpha (TNF-α) [4, 5]. IL-6 is produced by many different cell types including monocytes/macrophages, fibroblasts, endothelial cells and adipocytes. It regulates production of adhesion molecules involved in the release of other cytokines and induces the hepatic synthesis of C-reactive protein (CRP). *In vivo* release of IL-6 from human adipose tissue (AT) has been demonstrated [6]. Furthermore, positive associations between IL-6 and TNF-α in AT and circulating CRP have been shown [7]. TNF-α is synthesized by cells of the immune system and it is a strong mediator of inflammatory and immune functions [5]. Furthermore, it is known to regulate growth and differentiation of many different cell types. Macrophages in AT can produce TNF-α shown to be associated with proinflammatory activity that may contribute to atherosclerosis [8]. IL-1β is produced by several cell types including macrophages and it is a potent inducer of fever and the acute phase response [5]. The positive correlation seen between proinflammatory cytokines in AT and circulating CRP is of particular interest as it has been shown that decreased AT macrophage infiltration was associated with an improved inflammatory profile following weight loss in obese subjects [9]. Moreover, a positive association between body mass index and death from cardiovascular disease has been demonstrated [10], indicating that AT proinflammatory cytokines are pathogenic in atherosclerosis.

To elucidate a possible connection between systemic inflammation and inflammatory activity in AT in humans, we investigated whether a standardized inflammatory stimulus activates AT and circulating peripheral blood mononuclear cells (PBMC). To standardize inflammation, we used a model of vaccination against *Salmonella typhi*. Current knowledge about stimulated inflammatory activity in different cell types and tissues is mostly based on animal and *in vitro* studies, therefore it is of particular interest to investigate this in humans. Our hypothesis was that an acute systemic inflammation would stimulate inflammatory activity in AT that could sustain the systemic inflammatory response.

Eighteen healthy volunteers (16 men and two postmenopausal women) who had participated in two previous studies were invited (Table 1). Every second individual was allocated to the vaccine or control group. There have been conflicting data as to whether genetic variation in the IL-6 gene could explain variability in stimulated plasma levels of IL-6 [11–13]. Results from our group have clearly demonstrated that the G-allele of the 174 G>C polymorphism in the promoter region of the IL-6 gene is associated with increased IL-6 levels in plasma after vaccination [14]. To avoid differences in inflammatory response due to this polymorphism all subjects in this study were homozygous for the common −174 G allele. All subjects gave informed written consent for participation in the study which was approved by the Ethics Committee of the Karolinska Institutet.

Venous blood samples were obtained after 0, 4, 8, 12 and 24 h. After the initial blood sample, subjects

Table 1 Demographic data and basic characteristics

Variable	Vaccine group (n = 9)	Control group (n = 9)	P-value
Age, years	59 (58–64)	60 (59–63)	NS
Sex (men/women)	8/1	8/1	NS
Current smokers, n (%)	2 (22)	1 (11)	NS
Body weight, kg	93 (75–100)	90 (81–94)	NS
BMI, kg m^{-2}	25 (24.5–27.2)	28.2 (25.6–29.1)	NS
Waist circumference, cm	94.5 (84.3–103.5)	96 (91–100.5)	NS
Blood pressure, mmHg			
Systolic	140 (125–140)	140 (120–140)	NS
Diastolic	90 (80–95)	80 (70–85)	NS
Heart rate, bpm	64 (58–72)	60 (52–64)	NS
Plasma glucose, mmol L^{-1}	5.5 (5.2–5.7)	5.1 (4.9–5.7)	NS
Plasma cholesterol, mmol L^{-1}	5.0 (4.6–5.1)	5.9 (5.2–6.0)	NS
Plasma LDL, mmol L^{-1}	3.3 (3.2–3.5)	3.8 (3.1–4.5)	NS
Plasma HDL, mmol L^{-1}	1.1 (0.9–1.2)	1.3 (1.0–1.5)	NS
Plasma triglycerides, mmol L^{-1}	1.4 (1.0–1.5)	1.0 (0.9–2.7)	NS

Data are presented as median (interquartile range) and n (%). BMI, body mass index; NS, not significant.

in the vaccine group received an injection with vaccine against *S. typhi* (Typhim Vi; Sanofi Pasteur MSD, Solna, Sweden), 0.5 mL (25 μg), intramuscularly in the left shoulder. Four hours after the first blood sampling all subjects underwent a subcutaneous fat biopsy from the periumbilical area of the abdomen, as described previously[15]. The biopsy of 300–500 mg was washed in saline and immediately frozen in RNAlater (Ambion, Inc., Austin, TX, USA) at −70°C for gene expression studies. At 0 and 4 h venous blood samples were obtained for analysis of gene expression. These blood samples were taken in cell preparation tubes (BD, Franklin Lakes, NJ, USA) for separation of PBMC from whole blood. IL-6 in EDTA plasma was analysed by a high sensitivity enzyme immunoassay (R&D Systems Inc., Minneapolis, MN, USA).

Total RNA was extracted from PBMCs using the QIAamp RNA blood mini kit (Qiagen GmbH, Hilden, Germany). RNA was extracted from fat biopsies using the RNeasy mini kit (Qiagen). An Agilent 2100 Bio analyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to confirm the quality of extracted RNA. An ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to analyse the concentration of RNA. Three hundred nanograms of RNA from each sample was reverse transcribed to complementary DNA (cDNA) using the Invitrogen superscript first strand synthesis system for real time polymerase chain reaction (RT-PCR) using the random primers (Invitrogen Corporation, Carlsbad, CA, USA). To investigate which house keeping gene to use, cDNA from PBMCs from four of the subjects from the vaccine group were analysed before and 4 h after vaccination using the TaqMan Human Endogenous Control Plate as described (Applied Biosystems, Foster City, CA, USA). Cyclophylin A was stable during inflammatory stimulation and had a suitable Ct-value. To analyse gene expression 3 μL of cDNA was mixed with TaqMan universal PCR master mix (2×) (Applied Biosystems; Branchburg, NJ, USA) and primer-probe mix (20×) with Taqman gene expression assays (Applied Biosystems) to a final volume of 25 μL. The gene expression assays used for quantitative RT-PCR (TaqMan®) were cyclophylin A (Hs99999904_m1), IL-1β (Hs00174097_m1), IL-6 (Hs00174131_m1) and TNF-α (Hs00174128_m1).

Gene expression results for IL-1β, IL-6 and TNF-α were calculated as an index in relation to the cyclophylin A gene expression in each sample.

The results showed that in PBMCs, there was a significant increase in TNF-α mRNA gene expression 4 h after vaccination when compared with the control group ($P = 0.047$). There were no differences in mRNA gene expression of IL-1β and IL-6 between the vaccinated group and controls (Fig. 1a). In AT there were no differences in mRNA gene expression of IL-1β, IL-6 or TNF-α between the vaccine and the control group (Fig. 1b). The gene expression of IL-6 was higher in AT compared with PBMC ($P = 0.003$) and the gene expression of IL-1β was higher in PBMC compared with AT ($P = 0.0002$) taking the vaccinated group and controls together.

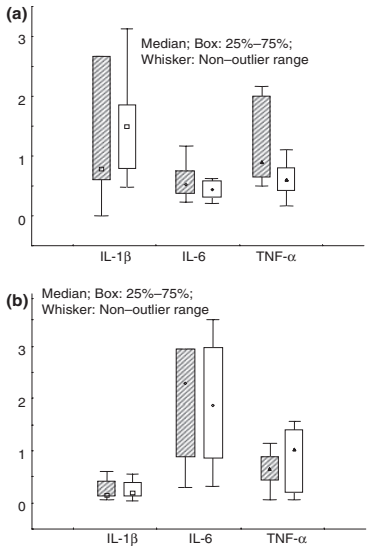

Fig. 1 RNA expression of inflammatory cytokines in relation to house keeping genes. (a) Peripheral blood mononuclear cells (PBMC). (b) Adipose tissue. Indexes of quantitative RNA gene expression of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) in relation to the house keeping gene cyclophylin A. Vaccinated subjects in striped boxes and controls in open boxes. Data are presented as median; box, 25–75%; whisker, nonoutlier range.

Plasma levels of IL-6 were higher in the vaccine group 8 h after vaccination, 4.86 ± 9.3 pg mL^{-1} compared with 1.6 ± 2.0 pg mL^{-1} in the control group ($P = 0.02$). (Fig. 2).

Previous studies on the inflammatory response after vaccination have not been able to measure any increase in plasma levels of TNF-α [14, 16]. The reason for this is not clear but one can speculate that the increased TNF-α expression in PBMCs seen in the present study is due to activated monocytes/macrophages released from the vaccinated tissue. The upregulation of TNF-α gene expression might not be enough to result in detectable plasma levels of the protein but the increased plasma levels of IL-6 support the experimental model of vaccination as a stimulus to systemic inflammation. The lack of upregulation of IL-6 gene expression in PBMCs 4 h after vaccination is likely to be due to differentiation of monocytes to macrophages of the subset of PBMCs with IL-6 gene expression thereby being trapped at the site of inflammation. According to our results TNF-α is a more relevant stimulus than IL-1β in stimulating IL-6 thereby extending previous studies in animals and *in vitro* [4]. One previous study has shown *in vivo* release of IL-6 from human subcutaneous AT [6]. However, the regulation of this synthesis is largely unknown. Our results support this study by demonstrating unstimulated IL-6 gene expression in AT. Interestingly, the basal level of mRNA gene expression was fourfold higher in AT compared with PBMCs. However, we were not able to

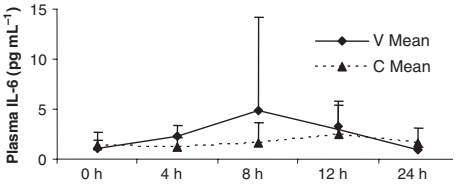

Fig. 2 Plasma levels of interleukin-6 (pg mL^{-1}). Vaccinated group in squares and controls in triangles. Data are presented as mean + SEM.

stimulate AT by vaccination. In contrast, Hoch *et al.* demonstrated increased transcription of IL-6 mRNA in human adipocytes stimulated *ex vivo* by lipopolysaccharides [17]. The reason for this discrepancy might be that we used a weaker stimulus to systemic inflammation. Furthermore, Memoli *et al.* showed that patients with chronic inflammation had increased IL-6 expression in AT when compared with patients with normal levels of inflammatory markers [18], suggesting that AT might respond to stimulation. Further *in vivo* studies with stronger stimulus to inflammation are needed to resolve this issue.

Plasma levels of IL-6 in our study increased only fourfold compared with basal values. Previous results from our group have shown that plasma IL-6 levels increased 10-fold after vaccination [14]. Compared with this study, subjects did not have an indwelling peripheral vein catheter during the study, otherwise the protocol was similar. The absence of an indwelling catheter could probably explain the difference in peak plasma IL-6 levels. To avoid the risk that a haematoma caused by the fat biopsy would stimulate systemic inflammation, we compared vaccinated subjects with nonvaccinated subjects instead of using subjects as their own controls. Interestingly, the biopsy did not seem to stimulate systemic inflammation. One limitation of the present study is that fat biopsy was taken only once, at 4 h. The reason for the time chosen for fat biopsy was that we have shown that circulating IL-6 starts to increase 4 h after vaccination [14]. We cannot exclude that fat biopsy at other time-points would have resulted in a different result.

In conclusion, we have shown that vaccination, a human model of inflammation, which causes endothelial dysfunction and activates coagulation [16, 19], stimulates a mild systemic inflammation but does not trigger proinflammatory gene expression in AT. Further *in vivo* studies including a stronger stimulus to inflammation are needed to elucidate the inflammatory capacity of AT.

Conflict of interest statement

None.

Acknowledgements

We would like to thank Camilla Hage at the Department of Cardiology, Karolinska University Hospital Solna, for excellent technical assistance. This work was supported by grants from the Swedish Heart Lung Foundation.

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II

Am J Physiol Endocrinol Metab 299: E234–E240, 2010.
First published May 18, 2010; doi:10.1152/ajpendo.00115.2010.

Systemic inflammation activates the nuclear factor-κB regulatory pathway in adipose tissue

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Submitted 17 February 2010; accepted in final form 14 May 2010

Ekström M, Halle M, Bjessmo S, Liska J, Kolak M, Fisher R, Eriksson P, Tornvall P. Systemic inflammation activates the nuclear factor-κB regulatory pathway in adipose tissue. *Am J Physiol Endocrinol Metab* 299: E234–E240, 2010. First published May 18, 2010; doi:10.1152/ajpendo.00115.2010.—Adipose tissue (AT) is a store of energy but also an endocrine organ with the capacity to produce and release proinflammatory mediators into the circulation. The mechanism that may trigger human AT inflammation on a cellular level still remains largely unknown. The aim of this study was to investigate whether an acute systemic inflammation increases AT inflammatory activity, focused on innate immunity. Open heart surgery results in an extensive acute systemic inflammation. Therefore, we investigated the in vivo gene expression and production of inflammatory mediators in omental and subcutaneous AT stimulated by surgery. Biopsies from omental and subcutaneous AT were collected before and after cardiopulmonary bypass. Blood samples were collected at the same time as the AT biopsies and plasma IL-6 levels were measured with ELISA. RT-PCR was used for quantification of relative AT gene expression. To verify the gene expression results on a protein level, we used immunohistochemistry and microdialysis. After surgery, in both omental and subcutaneous AT, there was a strong upregulation of nuclear factor-κB-regulated genes, e.g., chemokine ligand-2, E-selectin, IL-1β, IL-6, IL-8, and Toll-like receptor-2. Immunohistochemistry showed staining for E-selectin associated with a high number of macrophages in close contact with and in the vascular wall. Increased levels of IL-6 were detected in microdialysate from subcutaneous AT. In conclusion, we present the novel finding that this model of inflammation induced a strong inflammatory response in both omental and subcutaneous AT including adhesion of macrophages to an activated endothelium and release of IL-6 from AT interstitium. It can be hypothesized that AT exerts a modulatory effect on innate immunity in humans.

coronary artery bypass grafting

ADIPOSE TISSUE (AT) is not only a store of energy but an endocrine organ with capacity to produce and release proinflammatory mediators into the circulation. Hence, obesity is an inflammatory disease, demonstrated by increased levels of cytokines, such as interleukin (IL)-6 in the circulation, due to increased expression and synthesis of proinflammatory cytokines in AT (7, 15, 23). Since obesity is becoming a global epidemic, it is also an increasingly important riskfactor for cardiovascular disease (CVD) (22, 24). The link between AT and inflammation has been demonstrated by an in vivo release of IL-6 from human AT (19), and it has been estimated that ~30% of total circulating IL-6 is produced by AT (30). A prospective study has demonstrated a marked reduction in circulating levels of C-reactive protein (CRP) in patients undergoing weight loss surgery (13), and weight loss is also associated with decreased AT macrophage infiltration and an improved proinflammatory profile (3). Ex vivo experiments using human cultured adipocytes stimulated with lipopolysacharide (LPS) have demonstrated an induction of the nuclear factor-κB (NF-κB) regulatory pathway (4, 28). LPS has also been found to induce AT inflammation in vivo, demonstrated by increased gene expression of inflammatory mediators in subcutaneous AT (1, 17, 25). Despite showing an AT inflammation in these studies, the mechanism that may trigger AT to inflammation in vivo still remain largely unknown. Since the current knowledge about the capacity of AT to produce inflammatory markers is based on ex vivo studies or LPS-stimulated subcutaneous AT inflammation studied on a gene expression level, further in vivo studies including other stimuli of inflammation, focusing on both gene expression and protein production in both omental and subcutaneous AT, are needed to fully elucidate the AT inflammatory response. Open heart surgery results in an extensive acute systemic inflammation with high plasma levels of both IL-6 and tumor necrosis factor (TNF) (2), and it has also been shown to increase gene expression of inflammatory mediators as IL-1β, TNF, and Toll-like receptor (TLR)-2 and -4 in circulating leukocytes, possibly due to contact of circulating blood with the synthetic surface of the cardiopulmonary bypass (CPB) system (6, 21).

We hypothesized that open heart surgery would increase the inflammatory activity in AT. Therefore, we investigated the in vivo gene expression and production of inflammatory mediators, focusing on innate immunity in both omental and subcutaneous AT in patients undergoing open heart surgery with CPB. We present the novel finding that this model of inflammation induced a strong inflammatory response in both omental and subcutaneous AT including adhesion of macrophages to an activated endothelium and release of IL-6 from AT interstitium.

METHODS

Subjects. Patients were eligible if they had planned for elective coronary artery by-pass surgery and/or aortic or mitral valve replacement according to a standard surgical procedure at the Department of Thoracic Surgery at the Karolinska University Hospital, Solna, Sweden. Patients were excluded if they had unstable CAD or were being treated with corticosteroids. All together, 16 patients who had planned for open heart surgery underwent blood sampling and AT biopsies for gene expression before and after CPB (Table 1). All subjects provided

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Table 1. *Basic characteristics*

Characteristics	$n = 18$
Variable	
Age, yr	71.5 (43–86)
Sex (men/women)	18/0
Current smokers, *n*	1/18 (6%)
Former smokers	8/18 (44%)
Body weight, kg	82.9 (62.5–100.4)
BMI, kg/m^2	27.7 (21.1–33.2)
CPB, min	106 (50–221)
Time between *samples 1* and *2*, min, $n = 14$	125 (90–285)
History of diabetes	3/18 (17%)
Current medication	
Acetylsalicylic acid	11/18 (61%)
β-Blocker	12/18 (67%)
ACEi	8/18 (44%)
ARBs	4/18 (22%)
Calcium antagonists	5/18 (28%)
Diuretics	10/18 (56%)
Nitrates	5/18 (28%)
Statins	12/18 (67%)

Data presented as median (min and max values), numbers, and percentages. BMI, body mass index; CPB, cardiopulmonary bypass; ACRi, angiotensin-converting enzyme inhibitor; ARBs, angiotensin receptor blockers.

written informed consent to participate in the study, and the study protocol was approved by the Ethics Committee of the Karolinska Institutet.

AT biopsies. Biopsies of ~1 cm^3 from omental and subcutaneous AT were collected before institution of CPB and at 15–20 min after removal of the aortic cross-clamp when the patient had been weaned off CPB. The omental AT biopsies were taken through a small opening in the abdomen in the bottom of the wound, and the subcutaneous AT biopsies were taken deeply from the side of the median sternotomy incision.

Plasma IL-6 analysis. Blood samples to measure the plasma levels of IL-6 were collected through the indwelling radial artery catheter at the same time that the AT biopsies were taken. All blood samples were centrifuged at room temperature, whereafter plasma was separated and stored at −80°C. IL-6 in EDTA plasma was analyzed in duplicates using one Quantikine Human IL-6 immunoassay plate (R&D Systems, Minneapolis, MN). Intra-assay coefficient of varation was 10.2%.

Total RNA and cDNA preparation. Biopsies from omental and subcutaneous AT (omental 100–310 mg, subcutaneous 120–360 mg) were immediately placed in RNAlater (Ambion, Austin, TX) and then frozen at −80°C according to the manufacturer's instructions. Frozen AT was homogenized and total RNA extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the supplier's instructions, including a DNase digestion step (RNase-Free DNase set, QIAGEN) to remove any contaminating genomic DNA. An Agilent 2100 Bio analyzer (Agilent Technologies, Santa Clara, CA) was used to confirm the quality of extracted RNA. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to analyze the concentration of RNA. Average yields of total RNA were 3.0 (1.5–5.5) μg/100 mg omental AT wet wt and 2.2 (0.8–5.4) μg/100 mg subcutaneous AT wet wt. Three hundred nanograms of RNA from each sample was reverse-transcribed to complementary DNA (cDNA) by Invitrogen's superscript first-strand synthesis system for real-time RT-PCR using random primers (Invitrogen, Carlsbad, CA).

Gene expression studies. To investigate which housekeeping genes to use, cDNA from omental AT from four subjects was analyzed using TaqMan Human Endogenous Control Plate (Applied Biosystems, Foster City, CA). To analyze AT gene expression, cDNA was mixed with 2× TaqMan Universal PCR master Mix (Applied Biosystems, Branchburg, NJ) in a total volume of 90 μl, and all samples were run in duplicates. The samples were loaded onto a Low Density Assay (Applied Biosystems) including primers for four different housekeeping genes: eucaryotic 18S ribosomal RNA (Hs99999901_m1), β-actin (ACTB, Hs99999903_m1), β2-microglobulin (B2M, Hs99999907_m1), and peptidylpropyl isomerase A (PPIA, Hs99999904_m1 [cyclophillin A]). Primers for the other gene expression assays (Applied Biosystems) are listed in Table 2. Relative quantification (RQ) of gene expression was calculated with cyclophyllin A as the housekeeping gene, with the first biopsy in every paired analysis as a reference. All four housekeeping genes showed stability during inflammation in the endogenous control plate experiment described above. As cyclophyllin A also demonstrated a similar cycle threshold (C_T) value to the genes of interest, it was used for the RQ analyses.

Immunohistochemical staining of adipose tissue sections. The gene expression results were confirmed by immunohistochemistry in five patients [men, age 74 (46–86) yr, BMI 26.8 (25.2–33.2) kg/m^2, CPB 87 (74–186) min], time between first and second AT biopsy 125 (110–210) min. Immunohistochemical staining was performed on biopsies from subcutaneous AT to investigate active NF-κB-p65, the protein of E-selectin (SELE), and the presence of macrophages. Staining was performed using a standard protocol on serial sections from formalin-fixed paraffin-embedded sections. Five-micrometer-thick serial sections were first deparaffinised and then boiled in TE buffer (10 mM Tris·Cl, 1 mM EDTA, PH 7.5) at 98°C for 30 min. After blocking with goat serum (Vector Laboratories, Burlingame, CA), sections were incubated with primary antibodies at 4°C overnight. Primary antibodies were rabbit polyclonal anti-NF-κB-p65 (1:400; GeneTex, Irvine, CA); mouse monoclonal anti-CD62E (SELE) (1:25; Novocastra Laboratories, Newcastle, UK), and mouse monoclonal anti-CD68 (macrophages) (1:200, Novocastra Laboratories). After a wash in PBS buffer, sections were incubated with secondary biotinylated goat anti-mouse (1:2,000; DAKO North Amer-

Table 2. *Assays for RT-PCR*

Genes	Assay Number
Chemokine ligand 2 [CCL-2 (MCP-1)]	Hs00234140_m1
Interferon-β (INFβ)	Hs01077958_s1
Interferon-γ (INFγ)	Hs00174143_m1
Interleukin-10 (IL-10)	Hs00961619_m1
Interleukin-15 (IL-15)	Hs99999039_m1
Interleukin-18 (IL-18)	Hs01038788_m1
Interleukin-1β (IL-1β)	Hs00174097_m1
Interleukin-6 (IL-6)	Hs00174131_m1
Interleukin-8 (IL-8)	Hs00174103_m1
E-selectin (SELE)	Hs00174057_m1
P-selectin (SELP)	Hs00927900_m1
Tumor necrosis factor-α (TNF-α)	Hs00174128-m1
FOS	Hs00170630_m1
Intercellular adhesion molecule 1 (ICAM-1)	Hs00164932_m1
Interleukin-1 receptor type 1 (IL-1R1)	Hs00168392_m1
Interleukin-6 receptor (IL-6R)	Hs00174360_m1
JUN	Hs99999141_s1
Mitogen-activated protein kinase-3 (MAP3K)	Hs00394890_m1
Mitogen-activated protein kinase-4 (MAP4K)	Hs00377415_m1
Nuclear factor-κB 1 (NF-κB1)	Hs00231653_m1
Nuclear factor-κB 2 (NF-κB2)	Hs00174517_m1
RELA (p65)	Hs00153294_m1
Toll-like receptor 2 (TLR-2)	Hs00152932_m1
Toll-like receptor 4 (TLR-4)	Hs00152939_m1
Tumor necrosis factor receptor superfamily, member 1A (TNF-RSF1A)	Hs00236902_m1
Tumor necrosis factor receptor superfamily, member 1B (TNF-RSF1B)	Hs00153550_m1
Vascular cell adhesion molecule 1 (VCAM-1)	Hs00174239_m1

Table demonstrates primers and assay numbers for the gene expression assays (Applied Biosystems).

ica, Carpinteria, CA) antibodies or biotinylated goat anti-rabbit (1:2,000, Vector Laboratories) antibodies. Staining was visualized using avidin-biotin peroxidise complex (Vector Laboratories) followed by 3.3′-diaminobenzidine tetrachloride (Vector Laboratories). All sections were counterstained with filtered Harris hematoxylin (Histolab, Gothenburg, Sweden) and visualized with light microscopy.

The number of positive cells was estimated in relation to the number of vessels in every paired biopsy. A semiquantitative scale from 0 to +++ (where 0 is no positive cells, + is <25% positive cells, ++ is 25–75% positive cells, and +++ is >75% positive cells) was used for the evaluation of staining for NF-κB-p65, SELE, and CD68. The evaluation was performed blindly by two independent investigators. The agreement between the different investigators was >90%. The discrepancy was never more than one scale step, and consensus was obtained by reevaluation.

Microdialysis protocol. Local production of IL-6 was studied by microdialysis in subcutaneous AT during open heart surgery with CPB in four patients [men, age 68 (49–73) yr, BMI 28.3 (23.2–29.6) kg/m^2, CPB 123 (90–184) min]. At start of surgery, a microdialysis catheter (CMA 71; CMA Microdialysis, Stockholm, Sweden) with a 100,000 Da cut-off and a membrane length of 30 mm was inserted subcutaneously under sterile conditions in the upper left quadrant of the abdomen without the use of local anesthetics. The catheter membrane was inserted guided by a splittable introducer (SI-2, CMA Microdialysis), whereafter the introducer was immediately drawn back and the catheter membrane was left uncovered in the tissue. The catheter was covered with a sterile dressing membrane and immediately connected to a microdialysis pump (CMA 107, CMA Microdialysis) and infused by Ringer-acetate (Fresenius Kabi, Uppsala, Sweden) with a flow rate of 1 μl/min. The dialysate was collected at 30-min intervals for 330 min. Collected dialysate up to 60 min after catheter insertion was not included in the statistical analysis because of the normal equilibrating and fluid filling time period of 1 h of the catheter system (5). In the first patient, sampling was performed from two parallel catheters. In three patients, a second microdialysis catheter membrane was inserted after 150 min, 3–4 cm apart from the first one, in an attempt to discriminate between a local inflammation due to the catheter and a secondary inflammatory activation of AT. The two membranes were then run in parallel. The dialysate was collected into microvials (CMA Microdialysis) and immediately placed on ice and stored at −80°C.

Dialysate analysis. Dialysate was analyzed for lactate concentrations by means of a photometric method (ISCUS, CMA Microdialysis) and for IL-6 levels using ELISA (Human IL-6 DuoSet, R&D Systems). Intra- and interassay coefficients of variations were 9.4 and 15.0%, respectively.

Statistical analysis. Data are presented as medians (min-max), means (min-max) or means ± SE or numbers.

Differences between paired samples were analyzed using the nonparametric Wilcoxon matched-pairs signed ranks test. Due to the number of genes analyzed, only gene expression results were considered significant for genes where all samples showed an increase.

A mixed linear model was used to evaluate the effect of microdialysis catheters over time on IL-6 in dialysate. Time was entered in the model as a categorical variable with the levels at 90, 120, 150, and 180 min. An interaction term was included in the model to test for heterogeneous differences between catheters during the four time points. Due to the sparse sample size, any assumptions of the dependency between measures within patient was kept at a minimum. Hence, a compound symmetry covariance structure was used to account for the within effects, time, and catheter. Compound symmetry assumes a common error term and that the dependency is the same between each pair or repeated measures. As diagnostic tools for checking the model assumptions, normal probability plots and residual plots were used. The diagnostic tests showed right-skewed residuals and an increase of variation with time; therefore, log-transformed IL-6 was used in the final analysis. The significance level was specified at 0.05.

RESULTS

Systemic inflammation. After ~30–40 min of surgery, blood sampling was started and the first biopsies from omental and subcutaneous AT were collected. CPB was started and patients underwent a second sampling of blood and AT biopsies after CPB had been turned off. Details of the procedure and characteristics of subjects are given in Table 1. Open heart surgery with CPB induced a 25-fold increase in plasma IL-6 levels 3.0 pg/ml (1.9–5.9) before and 79.8 pg/ml (31.5–190.8) after CPB (see Fig. 3*A*; $n = 14$, $P = 0.008$).

Gene expression data in omental and subcutaneous AT. Paired AT biopsies were taken from nine patients, whereof both omental and subcutaneous AT biopsies were from six patients, only omental AT biopsy from one patient and only subcutaneous AT biopsies from two patients. In these patients, fasting plasma glucose in median was 5.6 (4.6–8.6) mmol/l and the total need for rapid insulin to keep plasma glucose below 10 mmol/l during the first 24 h after start of surgery in median was 59 (14–126) units. RQ of gene expression was calculated in relation to the housekeeping gene cyclophyllin A because it demonstrated stability during inflammation and a similar cycle threshold value to the genes of interest.

IL-6, IL-8, and SELE showed a 100-fold increase in mRNA expression in omental AT, and in subcutaneous AT these genes increased even more, up to 200- to 400-fold. The pattern of increased proinflammatory chemokine and cytokine gene expression was similar in omental (Fig. 1*A*) and subcutaneous AT (Fig. 1*B*).

The mRNA expression of receptors and transcription factors in omental and subcutaneous AT are presented in Fig. 1, *C* and *D*. In omental AT there was increased mRNA expression of IL-1R1, NF-κB1, TLR2, TLR4, and TNFR-SF1A (Fig. 1*C*). A similar pattern was found in subcutaneous AT with increased mRNA expression of FOS, JUN, TLR2, and TNFR-SF1B (Fig. 1*D*).

Immunohistochemistry. To verify the increased mRNA expression at protein level, staining and localization of active NF-κB-p65 was investigated by immunohistochemistry in subcutaneous AT biopsies. There was a marked staining of NF-κB-p65 in AT following surgery. NF-κB-p65 was localized to adipocyte nucleus, endothelium, and macrophages (CD68-positive cells) as illustrated in Fig. 2, *A* and *B*. Furthermore, SELE stained strongly in the vascular endothelium after surgery (Fig. 2, *C* and *D*). Finally, we found a high number of CD68-positive cells (macrophages) in close contact with and in the vascular wall (Fig. 2, *E* and *F*). The data are summarized in Table 3.

IL-6 in microdialysate. Local production of IL-6 in subcutaneous AT was analyzed using microdialysis during and after open heart surgery. In total, IL-6 was measured in seven microdialysis catheters. Dialysate was collected every 30 min, and IL-6 increased in the dialysate from 14 (5–25) pg/ml at 90 min to a level of 147 (49–358) pg/ml at 5 h ($P < 0.05$). To discriminate between local inflammation and secondary activation of AT inflammation by surgery, we used a second catheter membrane inserted at the end of surgery, assuming that local catheter-induced inflammation would result in similar IL-6 dynamics in both catheters. In dialysate from the first

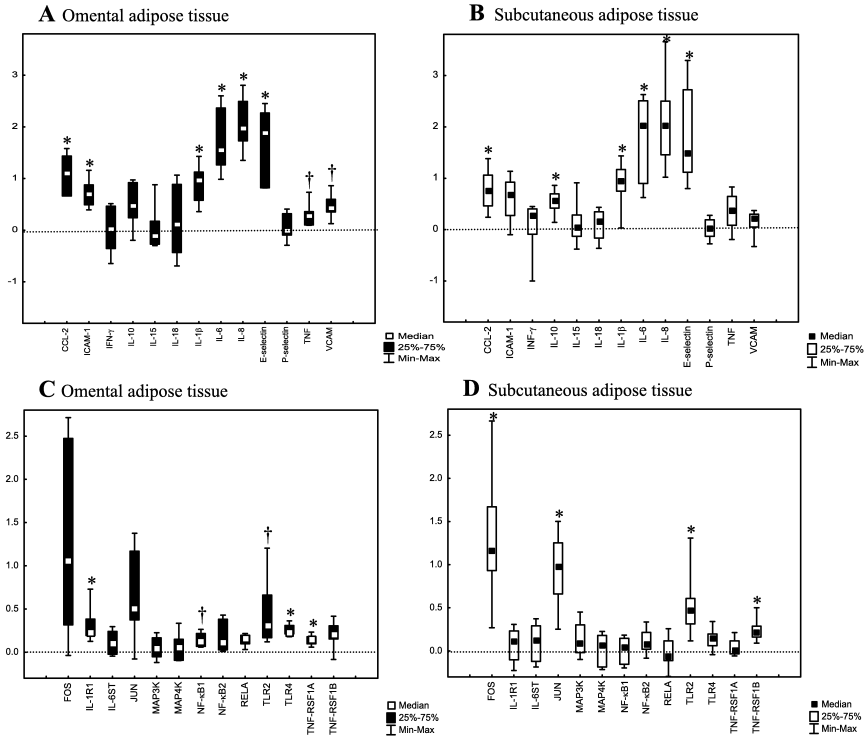

Fig. 1. Relative adipose tissue (AT) mRNA gene expression after open heart surgery expressed as change from baseline. Relative quantification (RQ) of mRNA gene expression of chemokines and cytokines (*A* and *B*) and receptors and transcription factors (*C* and *D*). RQ levels expressed in relation to the house keeping gene cyclophyllin A, using the first biopsy in every paired analysis as a reference and a logarithmic scale. Omental AT (7 patients) and subcutaneous AT (8 patients). $*P < 0.05$, $\dagger P < 0.02$.

microdialysis catheter ($n = 4$), there was an increased production of IL-6 approximately 3 h after start of surgery, whereas in dialysate from the second microdialysis catheter, inserted 2.5 h after the first catheter, an increased IL-6 production was already detected 90 min after insertion ($n = 3$). The mean levels of IL-6 in the second catheter were 2.3 times higher than the levels in the first catheter ($P = 0.005$; Fig. 3*C*). During this period, the lactate levels in dialysate were stable and did not increase in any of the patients (Fig. 3*B*).

DISCUSSION

To summarize the results, open heart surgery causes an increase in systemic IL-6 and an increased inflammatory gene expression in AT. First, gene expression of several proinflammatory genes increased dramatically after open heart surgery. Interestingly, the pattern was similar in both omental and subcutaneous AT. Second, the increased gene expression was evaluated with and supported by immunohistochemistry, showing strong staining of E-selectin with a high number of macrophages in close contact with and in the vascular wall. Third, increased gene expression of inflammatory mediators was followed by increased production of IL-6 in subcutaneous AT measured by microdialysis.

Several previous studies have demonstrated an inflammatory capacity of AT (1, 4, 8, 9, 12, 13, 15–20, 25, 26, 28), but in this in vivo study we present the novel finding that open heart surgery, a strong stimulus to systemic inflammation, induced upregulation of innate immunity gene expression in AT confirmed on a protein level by use of both immunohistochemistry and microdialysis. Previously, two ex vivo studies and three in vivo studies, using LPS as a stimulus to inflammation, have shown increased gene expression in AT (1, 4, 17, 25, 28). The present study not only confirms the results of those studies but extends the knowledge about AT inflammatory capacity by adding similar data from omental AT. Furthermore, our results from immunohistochemistry and microdialysis, done on a limited number of samples, both support that the increased gene expression ultimately results in protein production. Our results also suggest that the NF-κB regulatory pathway activates the AT inflammatory capacity. AT IL-6 content after operative trauma has been found to correlate with circulating levels of IL-6 (8). In our study, we found increased gene expression followed by IL-6 production in subcutaneous AT, measured by microdialysis, making it less likely that IL-6 in AT merely reflects plasma levels.

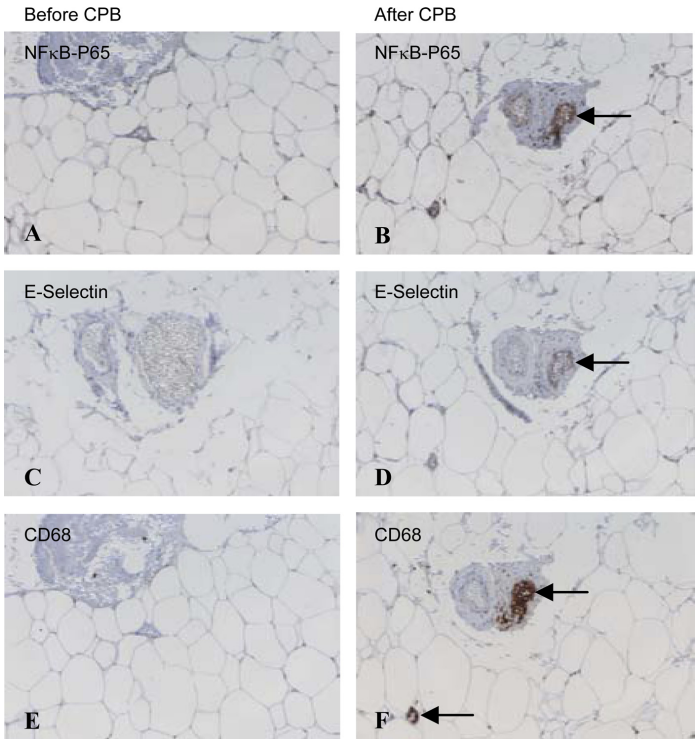

Fig. 2. Immunohistochemistry staining of subcutaneous AT. Representative micrographs of serial sections of subcutaneous AT before and after open heart surgery with cardiopulmonary bypass (CPB) demonstrating a marked upregulation of NF-κB-p65 (*A* and *B*) and a strong staining of E-selectin in the vascular endothelium (*C* and *D*) after CPB. There were also a high number of CD68-positive cells in close contact with the vascular endothelium migrating into the vessel wall (*E* and *F*). Arrows mark areas with strong positive staining.

Previous studies lack information about stimulated AT inflammation on a cellular level. Our results showed that several cell types within AT became activated by open heart surgery; immunohistochemistry showed activation of macrophages and endothelium (E-selectin) of AT blood vessels, whereas microdialysis from AT interstitium demonstrated increased IL-6 synthesis, to which, e.g., activated adipocytes, preadipocytes, and macrophages, could have contributed.

Microdialysis is a validated method to gain access to and sample adipose-derived molecules from the intercellular space, providing valuable information at a tissue level (5, 20, 27). These studies have indicated a risk of an artifact due to trauma from the catheter. Possibly, this risk may depend on which insertion technique and catheter membrane were used. To avoid this artifact risk, we used a two-catheter membrane protocol where the second membrane was inserted 2.5 h after the first membrane, assuming that local catheter-induced inflammation would result in similar IL-6 dynamics in both catheters. Our results showed an increased IL-6 production detected in dialysate from the first microdialysis catheter ~3 h after catheter insertion, but we can clearly demonstrate an increased IL-6 production in the second microdialysis catheter already at 90 min. This result confirms the findings from the gene expression experiment, and the NF-κB regulatory pathway might be the key through which IL-6 synthesis in adipocytes is regulated. However, a contribution of IL-6 from macrophages and endothelium in AT blood vessels cannot be excluded.

Table 3. *Results from immunohistochemistry*

	Before CPB				After CPB			
Group	0	+	++	+++	0	+	++	+++
E-selectin	0	5	0	0	0	0	2	3
NF-κB-p65	0	3	2	0	0	0	0	5
CD68	0	5	0	0	0	0	2	3

Table demonstrates evaluation results of immunohistochemical staining of subcutaneous adipose tissue sections from 5 subjects. A semiquantitative scale from 0 to +++ (0 = no positive cells, + = <25% positive cells, ++ = 25–75% positive cells, +++ = >75% positive cells) was used for evaluation of staining of E-selectin, NF-κB-p65. and CD68.

Open heart surgery results in an extensive acute systemic inflammation with high plasma levels of both IL-6 and TNF (2). This has previously been shown to be a result of gene expression of inflammatory mediators in circulating leukocytes (6, 21). Other sources of inflammatory mediators have not been investigated previously. One may speculate that circulating TNF activates the vascular endothelium and the surrounding AT to produce E-selectin and other inflammatory mediators through the NF-κB regulatory pathway. Secondary to the expression of E-selectin and adhesion molecules on the vascular endothelium, circulating monocytes are recruited and activated to differentiate into macrophages with the possibility to

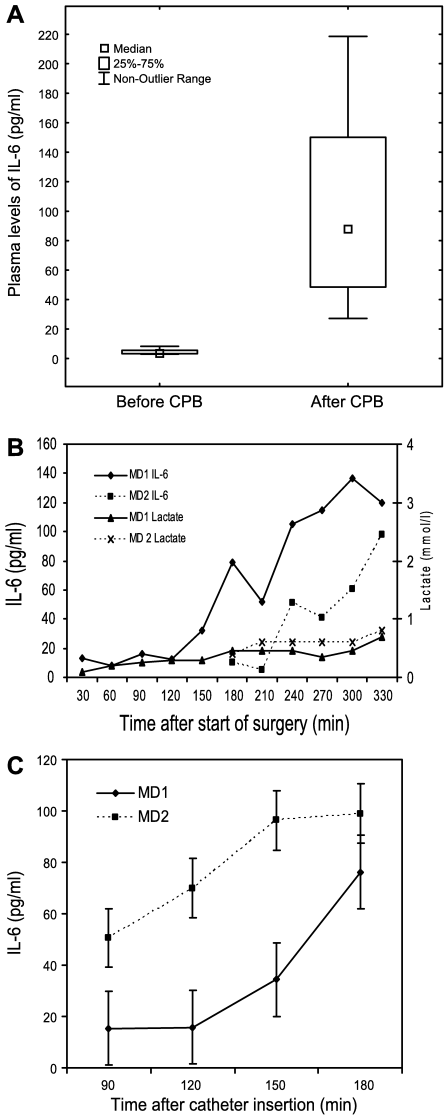

Fig. 3. Il-6 concentrations in plasma and IL-6 and lactate levels in microdialysate from subcutaneous AT. *A*: plasma levels of IL-6 before and after open heart surgery. There were increased plasma levels of IL-6 in paired samples taken before and after CPB ($P = 0.008$, $n = 14$). *B*: microdialysate from subcutaneous AT during open heart surgery. Median levels of IL-6 (pg/ml) and lactate (mmol/l), both microdialysis (MD) catheters from 30 to 330 min. *C*: IL-6 levels (pg/ml, mean ± SE) in microdialysate from subcutaneous AT during and after open heart surgery focused on the time period from 90 to 180 min after catheter insertion with both catheters in parallel. Dialysate from the 1st microdialysis catheter (MD1, $n = 4$) showed increased production of IL-6 ~3 h after start of surgery, and the 2nd microdialysis catheter (MD2, $n = 3$), inserted 2.5 h after MD1 demonstrated increased IL-6 production already after 90 min with 2.3× higher mean levels of IL-6 vs. MD1 ($P = 0.005$).

extravasate into the surrounding AT. This may be one explanation for the recruitment of macrophages to AT resulting in the low-grade chronic inflammation seen in obese subjects (3). Another explanation for recruitment of macrophages into AT in obese individuals is increased levels of free fatty acids that can induce NF-κB activation by TLR-4 followed by expression of cytokines, some of which suppress insulin signaling previously reviewed by Rocha and Libby (23). However, the mechanisms for triggering AT inflammatory capacity could differ in low-grade chronic and acute systemic inflammatory states.

It is already well documented that the injury inflicted by surgery evokes increased release of cytokines and stress hormones. A central effect of these mediators is the development of insulin resistance (14). Furthermore, previous studies have also shown improved glucose tolerance after neutralization of TNF in obese rodents (10, 11). Our results give further support to the importance of AT inflammatory capacity that might contribute to the acute systemic inflammation and thereby aggravate the increased insulin resistance during surgery. In these studies, this effect was transient and therefore may be of minor long-term clinical relevance, but if AT recruitment of macrophages during the acute phase of inflammation also contributes to the low-grade chronic AT inflammation seen in obese individuals, it may have a deeper impact on metabolic disorders and long-term clinical relevance. The human innate immune system has been developed during evolution as an acute-phase response to local or systemic infections. Increased AT may then have been beneficial in potentiating this response, thus serving as a survival benefit. However, this effect could have become disadvantageous with increasing adiposity, obesity-induced insulin resistance, and diabetes mellitus, predisposing to atherosclerosis and related CVD.

Our study has several limitations. One is the limited number of patients, another being only men, making it impossible to correlate the AT findings to the plasma inflammatory response. It would be of great interest to measure IL-6 during the perioperative as well as postoperative period in a substantial number of female and male patients to further investigate whether the inflammatory response to open heart surgery is stronger in overweight patients due to secondary activation of AT. Another limitation is the lack of data on insulin resistance. However, we found a positive correlation between the need for insulin during the first 24 h after start of surgery and relative quantification of IL-1β gene expression ($r = 0.86$, $P = 0.014$) and nonsignificant positive trends between the need for insulin and relative quantification of chemokine ligand-2 and IL-6 gene expression in omental AT after surgery. These data are supported by previous studies demonstrating that surgery causes a transient reduction in insulin sensitivity, reviewed by Ljungqvist et al. (14). However, due to the low number of study subjects, it is impossible to make any definitive conclusion from the correlation analyses. The need for insulin together with activation of AT could be investigated in a future study of the role of obesity during surgery.

In conclusion, we present the novel finding that open heart surgery, a model of inflammation, induced a strong inflammatory response in both omental and subcutaneous AT, including adhesion of macrophages to an activated endothelium and release of IL-6 from AT interstitium. It can be hypothesized that AT exerts a modulatory effect on innate immunity in humans.

ACKNOWLEDGMENTS

We thank Eva Wallgren at the department of Cardiology, Karolinska University Hospital, Solna, for excellent technical assistance and Jakob Bergstrom at the LIME Institution, Karolinska Institutet, for excellent support in medical statistics. We also thank the surgeons at the department of Thoracic Surgery at the Karolinska University Hospital for taking adipose tissue biopsies.

GRANTS

This work was supported by grants from the Swedish Heart Lung Foundation.

DISCLOSURES

No conflicts of interest are reported by the authors.

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III

Stimulated in vivo synthesis of plasminogen activator inhibitor-1 in human adipose tissue

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Key words: PAI-1, adipose tissue, inflammation, CABG

Abstract

Objective
Cardiovascular events are known complications after surgery and severe infection where acute systemic inflammation is a common denominator. To investigate a possible mechanism behind these complications we have studied plasminogen activator inhibitor-1 (PAI-1) synthesis in adipose tissue (AT) after acute systemic inflammation, induced by open heart surgery.

Methods
Twenty-two patients underwent blood sampling and omental and subcutaneous AT biopsies for gene expression studies before and after surgery. Expression and localization of PAI-1 in AT was evaluated by immunohistochemistry.

Results
After surgery, gene expression of PAI-1 increased 27-fold in omental AT and 3-fold in subcutaneous AT. PAI-1 antigen was localized within endothelial cells, in the AT interstitium close to AT vessels and in solitary cells between the adipocytes. The upregulated gene expression and protein synthesis in AT was followed by increased concentrations of PAI-1 antigen in plasma.

Conclusions
For the first time *in vivo*, we present that an acute systemic inflammation increased gene expression and protein synthesis of PAI-1 in human AT. The increase was most prominent in omental AT. PAI-1 synthesis in AT, following acute inflammation, may be a link between inflammation and impaired fibrinolytic activity that might explain the risk for myocardial infarction after surgery or infection.

Introduction

Cardiovascular events are known complications in up to five percent of patients undergoing non-cardiac surgery [1]. Furthermore, there is a five-fold increased risk of myocardial infarction during the first week after a severe infection [2, 3]. The mechanism behind this increased risk is largely unknown but an acute systemic inflammation is a common denominator of surgery and severe infection.

Fibrinolysis is a cascade of enzymatic processes leading to degradation of fibrin. This process is determined by both plasminogen activators and inhibitors, whereof PAI-1 is believed to be the most important inhibitor. Hepatocytes, platelets and vascular endothelial cells are believed to be the main producers of PAI-1, but the contribution of different tissues to circulating PAI-1 may differ in health and disease [4]. Previously, an association between adiposity and impaired fibrinolysis was observed [5, 6] and obese diabetic subjects are reported to have increased circulating concentrations of PAI-1 [7]. Importantly, both murine and human adipocytes have been shown to express PAI-1 mRNA [8-10].

The regulation of gene expression of PAI-1 in adipose tissue (AT) has been investigated in numerous studies. Proinflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF) increase PAI-1 mRNA in AT in animal models [8, 11, 12] while TNF, IL-1β and IL-6 all stimulate upregulation of PAI-1 gene expression in human adipocytes *ex vivo* [13, 14]. Other well-known inducers of PAI-1 synthesis in AT are angiotensin II, corticosteroids and insulin whereas catecholamines suppress PAI-1 gene expression and synthesis in AT [15-17]. The current knowledge regarding regulation of PAI-1 in AT is based on animal studies or *ex vivo* experiments on human adipocytes. However, it is unclear whether human adipocytes cultured *ex vivo* adequately represent the situation on a tissue level *in vivo*. So far, no study has described stimulated gene expression and protein synthesis of PAI-1 *in vivo* in human AT.

We hypothesized that an acute systemic inflammation, through the activation of AT inflammatory capacity, induces gene expression and protein production of PAI-1 *in vivo*. Both omental and subcutaneous AT were studied. To our knowledge, we present for the first time that an acute systemic inflammation in humans increased gene expression and protein synthesis of PAI-1 in AT and that this increase was more prominent in omental compared to subcutaneous AT. PAI-1 synthesis in AT due to acute systemic inflammation may be the link between inflammation and impaired fibrinolytic activity that might explain the increased risk of myocardial infarction seen after surgery or infection.

Methods

Subjects

Patients were eligible if they were planned for elective coronary artery by-pass (CABG) surgery and/or aortic or mitral valve replacement according to a standard surgical procedure at the Department of Thoracic Surgery at the Karolinska University Hospital, Solna, Sweden. Patients were excluded if they had unstable coronary artery disease or were treated with corticosteroids. Twenty-two male patients who were planned for open heart surgery underwent blood sampling and/or AT biopsies for gene expression and/or immunohistochemistry before and after cardiopulmonary bypass (CPB). Basic characteristics regarding all subjects are presented in Table 1. Plasma levels of PAI-1 during and up to six hrs after surgery were studied in seven patients (age 69 (43-78) yrs, BMI 27.7 (23.2-33.2) kg/m^2, CPB 102 (70-184) min). PAI-1 antigen staining intensity and localization in AT was determined by

immunohistochemistry in five patients (age 74 (46-86) yrs, BMI 26.8 (25.2-33.2) kg/m^2, CPB 87 (74-186) min), with time between first and second AT biopsy 125 (110-210) min. All subjects provided written informed consent to participate in the study and the study protocol was approved by the Ethics Committee of the Karolinska Institutet.

Table 1. Basic Characteristics

Variable	**N=22**
Age, yrs	69 (43-86)
Sex (men/women)	22/0
Current smokers, N (%)	1 (5%)
Former smokers, N (%)	9 (41%)
Body weight, kg	82 (69-100.4)
BMI, kg/m^2	27.4 (21.1-32.4)
CPB, min	94 (47-221)
Time between sample 1 and 2, min	125 (90-285)
Current medication	
Acetyl salicylic acid	14 (64%)
Beta blockers	15 (68%)
ACEi	8 (36%)
ARBs	4 (18%)
Calcium antagonists	5 (23%)
Diuretics	9 (41%)
Nitrates	5 (23%)
Statins	13 (59%)

Data presented as median (min and max values), numbers and percent. Body Mass Index (BMI), cardiopulmonary bypass (CPB), angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor lockers (ARBs).

Adipose tissue biopsies

Paired AT biopsies of approximately 1 cm^3 were taken from 13 patients, whereof both omental and subcutaneous AT biopsies from six patients, only omental AT biopsies from one patient and only subcutaneous AT biopsies from six patients. The AT biopsies were collected before institution of CPB and at 15-20 min after removal of the aortic cross-clamp when the patient had been weaned off CPB. The omental AT biopsies were taken through a small opening to the abdomen in the bottom of the wound and the subcutaneous AT biopsies were taken deeply from the side of the median sternotomy incision.

Plasma analysis of plasminogen activating inhibitor-1 and IL-6

Blood samples were collected in vacutainer ethylendiamid tetraacetic acid (EDTA) tubes

through an indwelling radial artery catheter at the same time as the AT biopsies or every hour up to six hrs after start of surgery. All blood samples were centrifuged in room temperature; where after plasma was separated and stored at –80ºC. PAI-1 antigen was analysed in duplicates using the DuoSet ELISA for human Serpine E1/PAI-1 (R&D Systems, Minneapolis, Minnesota, USA). Mean intra-assay, respectively inter-assay coefficient of variation (CV) were 6.5% and 5.1%. IL-6 was analyzed using Quantikine Human IL-6 Immunoassay (R&D Systems). Intra-assay CV was 10.2%.

Total RNA and cDNA preparation

Biopsies from omental (100-310 mg) and subcutaneous (120-440 mg) AT were immediately placed in RNAlater (Ambion, Austin, Texas, USA) and then frozen at –80ºC according to the manufacturer's instructions. Frozen adipose tissue was homogenized and total RNA extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the supplier's instructions including a DNase digestion step (QIAGEN) to remove any contaminating genomic DNA. An Agilent 2100 Bio analyzer (Agilent Technologies, Santa Clara, California, USA) was used to confirm the quality of extracted RNA. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) was used to analyse the concentration of RNA. Average yields of total RNA were 3.0 (1.5-5.5) µg per 100 mg omental AT wet weight and 2.5 (1.2-5.7) µg per 100 mg subcutaneous AT wet weight. Three hundred ng of RNA from each sample was transcribed to complementary DNA (cDNA) by Applied Biosystems cDNA-kit, using random primers (Foster City, California, USA).

Gene expression studies

To investigate which house keeping gene to use, cDNA from omental AT from four subjects was analysed using a TaqMan Human Endogenous Control Plate (Applied Biosystems). To analyse AT gene expression, cDNA was mixed with TaqMan® Universal PCR master Mix (Applied Biosystems) according to the manufacturer's instructions. RT-PCR was made using relative quantification with PAI-1 (Hs 00167155_m1) as the target gene and Cyclophylin A (Hs99999904_m1) as the endogenous control gene. Relative quantification of gene expression was calculated with Cyclophylin A as the house keeping gene and when differences in the degree of mRNA increase in omental and subcutaneous AT were analysed the first biopsy in every paired analysis was used as a reference. Cyclophylin A demonstrated stability during inflammation in the endogenous control plate experiment described above with a similar cycle threshold value (Ct-value) to the gene of interest.

Immunohistochemical staining of adipose tissue sections

Immunohistochemistry was performed on biopsies from omental and subcutaneous AT to investigate staining intensity and localization of PAI-1 antigen. Staining was performed using a standard protocol on serial sections from formalin-fixed paraffin-embedded sections. Four µm thick serial sections were first deparaffinised and rehydrated with ethanol. Antigen retrieval was achieved by microwave irradiation in EDTA buffer, pH 9.0. To block endogenous peroxidase activity, sections were treated with 0.3% H_2O_2 followed by serum block with 2% horse serum and an avidin-biotin blocking step (Vector Laboratories, Burlingame, California, USA). Hereafter, sections were incubated for 45 min with a monoclonal mouse anti PAI-1 antibody (GeneTex, Irvine, California, USA), diluted 1/20. A biotin-labelled horse anti-mouse antibody (Vector laboratories) containing 2% normal horse serum was used for detection. Phosphate buffer saline was used in all subsequent washes. All sections were developed

using a DAB-kit (Vector Laboratories) according to the instructions of the manufacturer. Sections were counterstained with Mayer's haematoxylin.
Evaluation of the immunohistochemical staining of PAI-1 included only subcutaneous AT. A semi-quantitative scale from 0 to +++ (where 0 is no positive cells, + is < 25% positive cells, ++ is 25-75% positive cells and +++ is > 75% positive cells) was used. The evaluation was performed blindly by two independent investigators. The agreement between the different investigators was > 90 %. The discrepancy was never more than one scale step and consensus was obtained by re-evaluation.

Statistical analysis
Data are presented as median (min-max), mean (min-max) or numbers (percent).
Skewed data were log transformed and differences analyzed using student's t-test. A test for linear trend was used to evaluate the differences in plasma levels of PAI-1 over time after surgery. The significance level was specified at <0.05.

Results

PAI-1 gene expression in AT
No difference was found in basal PAI-1 gene expression between the two AT depots. However, after open heart surgery there was increased mRNA expression of PAI-1 in both omental (p=0.028) and subcutaneous AT (p=0.002) (Figure 1). When these figures are analyzed as relative quantification using the first biopsy in every paired sample as a reference, the gene expression of PAI-1 in omental AT showed a 27-fold increase after surgery whereas a 3-fold increased gene expression of PAI-1 was found in subcutaneous AT. The degree of PAI-1 mRNA increase was higher in omental compared to subcutaneous AT (p=0.021).
Since angiotensin II is a strong inducer of PAI-1 synthesis in AT, we analyzed if the relative quantification of stimulated PAI-1 gene expression in subcutaneous AT was depending on treatment with angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs). Six out of twelve subjects were on ACEi/ARB medication all treated with maximal doses. However, we could not find any differences in PAI-1 gene expression between patients with or without angiotensin II blocking medication (data not shown).

Immunohistochemistry of PAI-1
To confirm inflammation-induced production of PAI-1 at a protein level and to investigate the localization of PAI-1 in AT, we stained for PAI-1 antigen in subcutaneous AT biopsies. There was a marked increased staining of PAI-1 antigen detected within the vascular endothelium as well as in the adventitia of AT vessels after open heart surgery. PAI-1 antigen was also detected in the AT interstitium close to AT vessels and in solitary cells in between adipocytes (Figure 2 and Table 2).

Plasma levels of IL-6 and PAI-1 during and after open heart surgery
To investigate circulating concentrations of IL-6 and PAI-1 antigen, blood samples were collected before and after open heart surgery with a median time of 125 (90-285) min between the paired samples. Open heart surgery with CPB induced a more than 25-fold increase in plasma IL-6 levels, in median 3.1 pg/ml (3.0-5.9) before and 85.2 pg/ml (31.5-218.2) after CPB (p<0.001, n=16). No significant differences were found in plasma levels of PAI-1 antigen analyzed before and after CPB (Figure 3a). In subjects further investigated

with plasma samples every hour up to six hrs after start of surgery, a positive linear trend was found in plasma levels of PAI-1 antigen with an increase after four hrs after start of surgery ($p<0.001$, n=7) (Figure 3b).

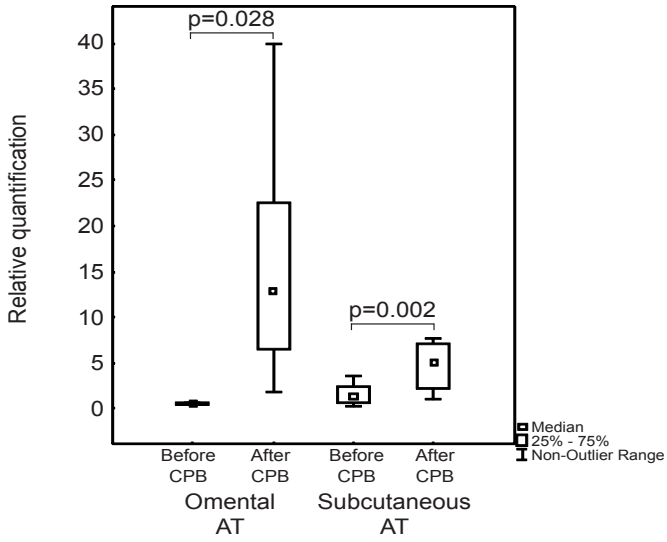

Figure 1. Relative quantification of gene expression of plasminogen activator inhibitor-1 (PAI-1) in omental and subcutaneous adipose tissue (AT) before and after open heart surgery.
Relative quantification of gene expression of PAI-1 in relation to the house keeping gene Cyclophylin A before and after cardiopulmonary bypass (CPB). No difference was found in basal levels of PAI-1 gene expression between the two different AT depots. After open heart surgery there was increased mRNA expression of PAI-1 in both omental ($p=0.028$, n=7) and subcutaneous AT ($p=0.002$, n=12). Median time between samples was 125 (90-285) min.

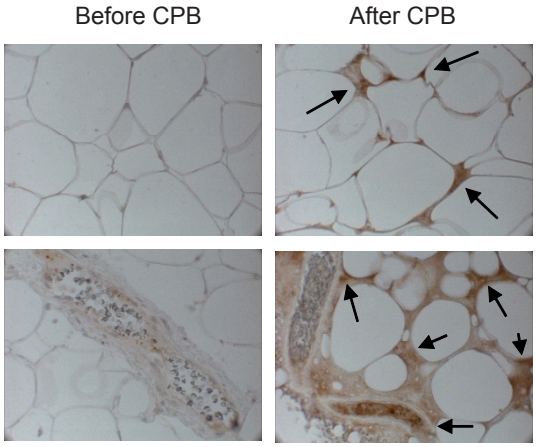

Figure 2. Localization of plasminogen activator inhibitor-1 (PAI-1) antigen in subcutaneous adipose tissue (AT) before and after open heart surgery.
Representative immunohistochemical analyses of paraffin sections (40x magnification) showing vasculature and adipocytes in subcutaneous AT. Strong staining of PAI-1 was detected within the endothelial cells as well as in the adventitia, after surgery. PAI-1 antigen was also detected in the AT interstitium close to AT vessels and in solitary cell nuclei in between adipocytes (n=5). Cardiopulmonary bypass (CPB).

Table 2. Immunohistochemistry of plasminogen activator inhibitor-1 (PAI-1) in subcutaneous adipose tissue biopsies

	Before CPB			After CPB		
	+	++	+++	+	++	+++
PAI-1	4	1	0	0	1	4

A semiquantitative scale from 0 to +++ (0 = no positive cells, + = < 25% positive cells, ++ = 25-75% positive cells, +++ = > 75% positive cells) was used for the evaluation of the staining of plasminogen activator inhibitor-1 (PAI-1). Cardiopulmonary bypass (CPB).

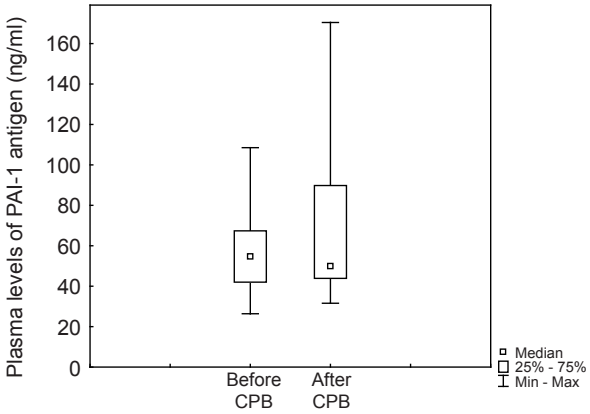

Figure 3a. Plasma levels of plasminogen activator inhibitor-1 (PAI-1) before and after open heart surgery.
No differences were found in plasma levels of PAI-1 antigen (ng/ml) analyzed before and after open heart surgery. Median time between samples was 125 (90-285) min (n=16).
Cardiopulmonary bypass (CPB).

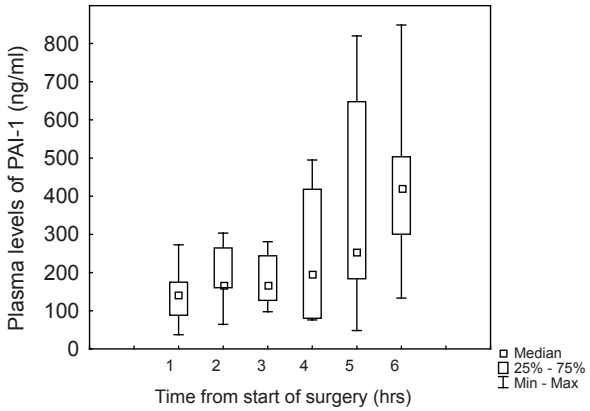

Figure 3b. Plasma levels of plasminogen activator inhibitor-1 (PAI-1) during and up to six hrs after start of open heart surgery.
A positive linear trend was found in plasma levels of PAI-1 antigen (ng/ml) with an increase after four hrs after start of surgery ($p<0.001$, n=7).

Discussion

In the present study we show that an acute systemic inflammation stimulates human AT to synthesise PAI-1 *in vivo*. Open heart surgery stimulates to a markedly increased gene expression of PAI-1 in AT, which is significantly higher in omental compared to subcutaneous AT. Stimulated PAI-1 protein is located within endothelial cells and adventitia as well as in AT interstitium close to AT vessels and in solitary cells in between adipocytes. And last, the upregulated PAI-1 gene expression and protein synthesis in AT was followed by an increased plasma level of PAI-1 after surgery.

The results of the present study may be questioned as being only a confirmation of earlier animal studies and *ex vivo* experiments on human adipocytes. However, our results are both new and of potential major clinical relevance. The increased risk of myocardial infarction seen after major surgery does not appear during the surgical procedure itself, characterized by the stress from anaesthesiology and surgical trauma, but appears during the early postoperative period [1]. The same phenomenon is found after a severe infection with the highest risk of myocardial infarction during the first week after onset of infection [2]. Both surgery and infection result in a systemic inflammation and the delay in time indicates that a putative risk factor for myocardial infarction is synthesized as a response to the acute phase reaction. From this point of view molecules that promote thrombus formation at a coronary artery plaque rupture, such as PAI-1, would be of major interest. Support for this hypothesis is that chronically elevated levels of PAI-1 are associated with spontaneous coronary arterial thrombosis in transgenic mice [18] and that increased activity of PAI-1 on the first day after CABG has been found to correlate to early vein graft occlusion [19]. However, the origin of this increased PAI-1 activity is still largely unknown. In this context, it is highly relevant to investigate the *in vivo* synthesis of PAI-1 in human AT during and after open heart surgery.

In the present study, we clearly demonstrate that human AT rapidly starts to increase the synthesis of PAI-1 as a response to the acute systemic inflammation and that the plasma levels of PAI-1 antigen increase after surgery, an increase to which the contribution of AT PAI-1 production could be highly relevant. This increase in plasma PAI-1 is not due to circadian variation since synthesis and plasma levels of PAI-1 is known to exhibit a circadian variation with peak levels at 6 am, thereafter the concentrations decrease in plasma [20]. Interestingly, our results show a markedly higher PAI-1 gene expression in omental compared to subcutaneous AT following surgery which underlines the importance of abdominal fat distribution in obesity. The intensified synthesis of PAI-1 in human AT following stimulation could severely impair the fibrinolytic activity and may therefore explain the risk of myocardial infarction seen after surgery or infection. The PAI-1 response in omental AT following stimulation could also be one of the explanations why obesity is an independent risk factor for myocardial infarction [21].

It is well known that open heart surgery is followed by an extensive acute systemic inflammation with high plasma levels of IL-6 and TNF [22, 23]. Our results confirm these data showing marked increased plasma levels of IL-6 already when the patients had been weaned off CPB. This acute response is explained by increased gene expression of inflammatory mediators like IL-1β, TNF, toll-like receptor-2 and 4 in circulating leukocytes, possibly due to a reaction between circulating blood and the synthetic surface of the CPB system [24, 25]. Previously, we have demonstrated that open heart surgery with CPB activates AT inflammation through the nuclear factor-κB regulatory pathway resulting in down-stream gene expression and synthesis of proinflammatory cytokines, chemokines and adhesion molecules [26]. Our results regarding increased gene expression of PAI-1 in AT, following open heart surgery indicate that PAI-1

synthesis in AT is regulated through the nuclear factor-κB regulatory pathway, congruent with a recent review by Kruithof [27] and supported by previous findings from animal studies [11] and *ex vivo* experiments on human adipocytes [9] where lipopolysacharide (LPS) or TNF were used as inflammatory stimuli. These characteristics make our model of inflammation comparable to LPS or TNF stimulation in animal studies or *ex vivo* studies on cultured human adipocytes and therefore give us the opportunity to investigate the *in vivo* effects of a strong acute systemic inflammation in humans.

Previously, *ex vivo* experiments have shown that angiotensin II stimulates PAI-1 gene expression in human adipocytes and that ACEi/ARBs were able to completely prevent this stimulation [17]. The use of angiotensin II inhibitors has also been reported to alter fibrinolytic activity during open heart surgery [28]. However, we could not find any differences in stimulated PAI-1 gene expression between patients with or without ACEi/ARBs treatment in our study. We can conclude that angiotensin II is not a likely major stimulatory factor of PAI-1 synthesis in AT during acute systemic inflammation.

In the present study, we demonstrate a markedly higher degree of upregulation of PAI-1 gene expression in omental compared to subcutaneous AT following an acute systemic inflammation. However, we found no significant differences in basal levels between the two AT depots. The results confirm earlier *ex vivo* studies by Alessi and Gottschling-Zeller who also demonstrated higher PAI-1 synthesis in omental versus subcutaneous cultured adipocytes following stimulation [9, 29]. These findings have been contradicted by another study demonstrating a lower *ex vivo* secretion rate of PAI-1 in omental compared to subcutaneous AT [30]. However, this study investigated PAI-1 production in non-stimulated conditions. Two additional studies have measured basal arterio-venous concentration differences of PAI-1 antigen across omental and subcutaneous AT but neither of them could show any significant differences across different AT beds *in vivo* [4, 31]. However, Yudkin and co-workers found a greater arterio-venous difference in PAI-1 activity in diabetic compared to nondiabetic subjects but the groups were small and the results were not paralleled by PAI-1 antigen concentrations. Nevertheless, the contribution of different AT beds to circulating PAI-1 could differ in health and disease.

We found a markedly increased gene expression of PAI-1 and a strong staining of PAI-1 antigen in AT biopsies taken already when the patient had been weaned off CPB, while plasma levels of PAI-1 did not increase until hours after surgery. This clearly suggests that the PAI-1 protein detected in AT by immunohistochemistry does not merely reflect plasma levels. It suggests that PAI-1 synthesis in AT significantly contributes to the later increase in plasma concentrations of PAI-1. In this context, our results emphasize the importance of omental compared to subcutaneous AT. The kinetics of PAI-1 synthesis within omental AT has never been investigated in detail but results from experiments on cultured human adipocytes showed increased synthesis of PAI-1 antigen five hrs after stimulation [9].

Limitations

In our study we only measured plasma concentrations of PAI-1 antigen and not PAI-1 activity or t-PA to which PAI-1 partly is found in complex. However, there is a close association between antigen levels and activity [32]. Furthermore, measuring both gene expression and protein levels of PAI-1 antigen could be more relevant than analyzing PAI-1 activity alone.

Another limitation is that there are other sources of plasma levels of PAI-1, such as activated platelets. However, PAI-1 in platelets is already synthesized and ready to be released whenever the platelets are activated. Most likely, an increased plasma level of PAI-1 due to activated platelets would have been detected earlier, possibly already during surgery. A third limitation

may be the low numbers of AT, in particular omental, biopsies and the potential risk of local tissue trauma when taking the biopsies. However, the use of paired biopsies before and after surgery minimizes the risk of inter-individual variation compensating for the low numbers. To avoid local stress from the surgical trauma the omental AT biopsies were taken through a small opening to the abdomen in the bottom of the wound and the subcutaneous AT biopsies were taken deeply from the side of the median sternotomy incision. A fourth limitation is the low number of subjects with plasma samples taken after surgery. However, our result of an increase in circulating PAI-1 after open heart surgery is supported by the study of Moor and co-workers who showed increased PAI-1 activity the day after surgery [19].

Our study clearly demonstrates that increased PAI-1 synthesis in AT during an acute phase response is one possible link between inflammation and myocardial infarction. Other systems important for coagulation and haemostasis may also react upon an acute systemic inflammation. Increased plasma levels of activated factor VII has been shown following a low grade acute inflammation [33] and interactions between bacterial pathogens and platelet receptors which result in platelet activation have also been demonstrated [6, 34].

In conclusion, we present for the first time *in vivo* that an acute systemic inflammation increased gene expression and protein synthesis of PAI-1 in human AT and that this increase was more prominent in omental compared to subcutaneous AT. PAI-1 synthesis in AT due to acute systemic inflammation may be the link between inflammation and impaired fibrinolytic activity that might explain the increased risk of acute myocardial infarction seen after surgery or infection.

Acknowledgements

We would like to thank Eva Wallgren at the department of Cardiology, Karolinska University Hospital, Solna, for excellent technical assistance and Jakob Bergstrom at the LIME-institution, Karolinska Institutet, for excellent support in medical statistics. We would also like to thank the surgeons at the department of Thoracic Surgery at the Karolinska University Hospital for taking AT biopsies.

Funding: This work was supported by grants from the Swedish Heart Lung Foundation.

Disclosure: None of the authors have any relationships that could be perceived as real or apparent conflict(s) of interest for this study.

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IV

Acute systemic inflammation does not affect adiponectin and leptin synthesis in humans

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Conflicts of interest: None
Relationships with industry: None

Abstract

Background
Adipose tissue (AT) is not only an energy-depot but also an organ producing inflammatory and metabolically active cytokines. Modulation of these adipokines during an acute inflammatory response is of considerate interest as they are suggested to have an important role also in chronic inflammation and in the development of atherosclerosis. However, the regulation of adipokines in the early phase of acute inflammation in humans is poorly understood. In this study acute inflammation was stimulated by vaccination and open heart surgery respectively, with the aim to investigate the effects on adiponectin and leptin synthesis.

Materials and methods
Adiponectin and leptin were measured as plasma levels after vaccination, respectively as by gene expression in omental and subcutaneous AT after surgery.

Results
Plasma levels of interleukin (IL)-6 increased 3-4-fold after vaccination whereas IL-6 increased 30-folds after open heart surgery. Neither plasma levels of adiponectin nor leptin were changed after vaccination. The gene expression levels of adiponectin or leptin were unaltered in both omental and subcutaneous AT after surgery.

Conclusion
We found no evidence that an acute systemic inflammation could affect synthesis of adiponectin or leptin indicating that these two adipokines are not key elements in the early acute-phase response.

Introduction

Adipose tissue (AT), classically thought to be merely a storage place for energy, has been shown to produce inflammatory and metabolically active cytokines. Chronic activation of innate immune responses within AT characterizes development of insulin resistance and atherosclerosis (1). Recently, adiponectin and leptin, two adipokines that primarily are synthesized by adipocytes, have attracted considerable attention because inflammation has been suggested to modulate adipokine levels. However, the regulation of adiponectin and leptin is complex and the knowledge about their synthesis within the early onset of inflammation still is poorly understood. Acute inflammatory effects on AT are, in this context, of particular interest because of the role of adipokines in the development of atheroscleroisis.

Adiponectin decreases with increasing obesity and has been described as anti-atherogenic including its positive effects on insulin sensitivity, whereas leptin is involved in the central regulation of appetite (1-3). A rapid weight loss in obese individuals decreases plasma levels of leptin while levels of adiponectin do not change (4). However, a longer period of weight loss and lifestyle changes increases adiponectin levels (5). Despite that plasma levels of leptin were increased and positively correlated with proinflammatory cytokines during sepsis (6) and further associated with increased survival of sepsis (7), few studies have addressed the influence of innate immunity on adiponectin and leptin in humans. The results of previous studies have been conflicting. One previous *in vitro* study has demonstrated that tumor necrosis factor (TNF) has dual effects on AT leptin release and synthesis with increased release of preformed leptin but inhibition of AT leptin synthesis (8). Anderson and coworkers have recently shown that leptin levels in plasma increased but there were no changes in adiponectin levels after lipopolysaccharide (LPS) injection *in vivo*. Moreover, on a gene expression level, only adiponectin was affected with suppressed mRNA expression in subcutaneous AT (9). Another study showed no effect of inflammation, caused by subarachnoidal hemorrhage, on adiponectin and leptin mRNA in subcutaneous AT (10). Methodological differences between these studies could partly explain differences in the results and therefore we don't know if adiponectin and leptin are important in the acute-phase response or how they are regulated. The present study is a sub-study to two earlier studies where we used two different models of inflammation: vaccination against salmonella typhi and open heart surgery, which both stimulate to an acute systemic inflammation through activation of the NF-κB regulatory pathway (11, 12).

Material and methods

Subjects

Eighteen healthy volunteers (sixteen men and two postmenopausal women) with a median age of 60 (49-67) years and BMI of 26.8 (19.8-38.8) were included in the vaccination study. Their basic characteristics have been described in detail (11) and are in brief presented in Table 1. Nine of the study subjects were vaccinated against Salmonella typhi (typhim Vi, Sanofi Pasteur MSD, Sweden), whereas the remaining subjects served as controls. Subjects arrived at 7 a.m. to the Karolinska University Hospital after fasting overnight. Venous blood samples were obtained after 0, 4, 8, 12 and 24 hrs. After vaccination and/or first blood sample participants had a light breakfast including a sandwich with cheese and a cup of coffee or tea with sugar and milk as preferred.

The patients in the open heart surgery study were eligible if they were planned for elective coronary artery by-pass (CABG) surgery and/or aortic or mitral valve replacement according to a standard surgical procedure at the Department of Thoracic Surgery at the Karolinska University Hospital, Solna, Sweden. Patients were excluded if they had unstable coronary

artery disease or were treated with corticosteroids. Nine male patients, with a median age of 65 (43-85) years and BMI of 27.7 (21.1-32.4), were included for the gene expression study and underwent AT biopsies and blood samples before and after cardiopulmonary bypass (CPB) as described (12). Basic characteristics are presented in Table 1.
All subjects provided written informed consent to participate in the study and the study protocol was approved by the Ethics Committee of Karolinska Institutet.

Table 1. **Basic characteristics.**

	Vaccination study		**Open heart surgery study**
Variable	Vaccinated n=9	Controls n=9	n=9
Age, yrs	59 (56-66)	60 (49-67)	65 (43-85)
Sex (men/women)	8/9 (89%)	8/9 (89%)	9/0
Current smokers, N Former smokers	2 (22%) 5 (56%)	1 (11%) 4 (44%)	1/9 (11%) 4/9(44%)
Body weight, kg	93 (60-127)	90 (64-109)	79.9 (62.5-92.7)
BMI, kg/m^2	25 (21-38.8)	28.2 (19.8-32.5)	27.7 (21.1-32.4)
CPB, min	NA	NA	98 (50-221)
Time between sample 1 and 2, min	NA	NA	125 (90-285)
History of Diabetes	NA	NA	1/9 (11%)
Current medication:			
Acetyl salicylic acid	NA	NA	7/9 (78%)
Beta blocker	NA	NA	2/9 (22%)
ACEi	NA	NA	2/9 (22%)
ARBs	NA	NA	2/9 (22%)
Calcium antagonists	NA	NA	3/9 (33%)
Diuretics	NA	NA	2/9 (22%)
Nitrates	NA	NA	4/9 (44%)
Statins	NA	NA	8/9 (89%)

Data presented as median (min and max values), numbers and percent.
No differences were found between vaccinated and controls in the vaccination study.
Angiotensin converting enzyme inhibitor (ACEi), angiotensin receptor blockers (ARBs), Body mass index (BMI), cardiopulmonary bypass (CPB), data not applicable (NA).

Plasma analyses
Plasma IL-6 sampling before and after vaccination and open heart surgery, respectively has been described in detail (11, 12). In brief, in the vaccination study venous blood samples were obtained after 0, 4, 8, 12 and 24 hrs, without an indwelling venous catheter. In the open heart surgery study the first blood sampling was made after approximately 30-40 min of surgery, before start of cardiopulmonary bypass (CPB). The second blood sampling was made after the CPB had been turned off with a median time of 125 (90-285) min between the paired samples. The blood samples in the open heart surgery study were obtained from an indwelling radial

artery catheter. All blood samples were collected in vacutainer ethylendiamid tetraacetic acid (EDTA) tubes and centrifuged in room temperature; where after plasma was separated and stored at –80ºC.
Plasma levels of adiponectin and leptin in the vaccination study were analyzed in duplicates using double-antibody radioimmunoassays (RIA) (Linco, St. Louis, MO, USA). CV for adiponectin was 15.2% at low (2–4 µg/mL) and 8.8% at high (26–54 µg/mL) levels. CV for leptin was 4.7% at both low (2–4 ng/mL) and high (10–15 ng/mL) levels.
Plasma levels of IL-6 in the vaccination study were determined in duplicates using a high sensitive ELISA (R&D Systems, Minneapolis, Minnesota, USA) with an intra-assay CV of 9.5%. Plasma levels of IL-6 in the open heart surgery study were analyzed in duplicates using one Quantikine Human IL-6 Immunoassay plate (R&D Systems) with an intra-assay coefficient of variation (CV) of 10.2%.

Adipose tissue biopsies
Paired AT biopsies of approximately 1 cm^3 were taken from nine patients, whereof both omental and subcutaneous AT biopsies from six patients, only omental AT biopsies from one patient and only subcutaneous AT biopsies from two patients. The AT biopsies were collected at the same time as the paired blood samples, before institution of CPB and at 15-20 min after removal of the aortic cross-clamp when the patient had been weaned off CPB. The omental AT biopsies were taken through a small opening to the abdomen in the bottom of the wound and the subcutaneous AT biopsies were taken deeply from the side of the median sternotomy incision.

Gene expression studies
The protocol for total mRNA and cDNA preparation has been described in detail (12). To investigate which house keeping gene to use, cDNA from omental AT from four subjects was analyzed using a TaqMan Human Endogenous Control Plate (Applied Biosystems, Foster City, California, USA). To analyze AT gene expression, cDNA was mixed with TaqMan® Universal PCR master Mix (Applied Biosystems) according to the manufacturer's instructions. RT-PCR was made using a custom made Low Density Array (Applied Biosystems) with Adiponectin (Hs00605917_m1) and Leptin (Hs00174877_m1) as target genes and Cyclophylin A (Hs99999904_m1) as an endogenous control gene, with a RT-PCR protocol according to the manufacturer's instructions. Relative quantification of gene expression was calculated with Cyclophylin A as the house keeping gene using the first biopsy in every paired analysis as a reference. Cyclophylin A demonstrated stability during inflammation in the endogenous control plate experiment described above with a similar cycle threshold value (Ct-value) to the gene of interest.

Statistics
Data are presented as median (min-max) or numbers (percent). Differences between continuous variables have been analyzed using Mann-Whitney U-test or Wilcoxon signed-rank test. The significance level was specified at <0.05.

Results

Systemic Inflammation
Plasma IL-6 levels after vaccination and open heart surgery have previously been reported (11, 12). After vaccination the plasma levels of IL-6 increased 3-4-fold with a significant difference at 8 hours after vaccination, while open heart surgery resulted in a 30-fold increase in plasma levels of IL-6.

Adipokines

Plasma levels of adiponectin and leptin were unaltered after vaccination, showing similar levels as in control group (Figure 1). Relative mRNA gene expression after open heart surgery, expressed as change from baseline was analyzed in both omental and subcutaneous AT biopsies. Neither adiponectin nor leptin mRNA from omental AT did change after open heart surgery with a median time of 125 (90-285) min between the biopsies. Similar results were obtained for subcutaneous AT (Figure 2).

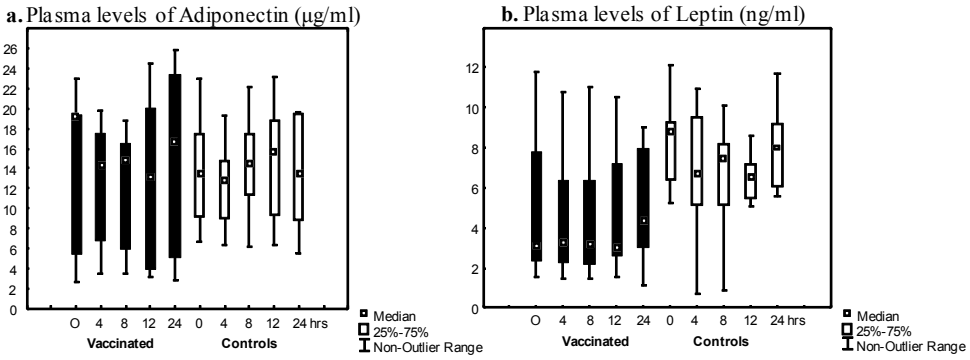

Figure 1. Plasma levels of adiponectin and leptin before and after vaccination

Box plots of plasma levels of adiponectin (a) and leptin (b) in healthy subjects vaccinated against Salmonella typhi (n=9, black boxes) and controls (n=9, open boxes).

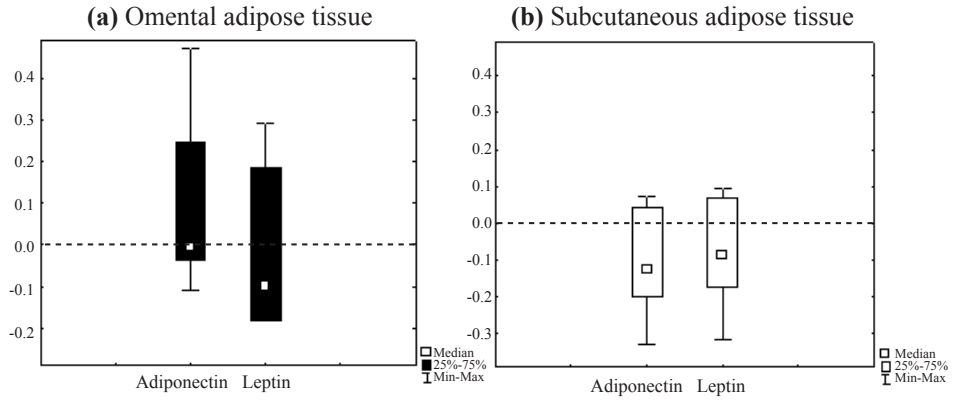

Figure 2. Relative adipose tissue mRNA gene expression after open heart surgery expressed as change from baseline

Relative quantification of adiponectin and leptin in (a) omental adipose tissue (AT) and (b) subcutaneous AT. Relative quantification expressed in relation to the house keeping gene Cyclophylin A, using the first biopsy in every paired analysis as a reference and a logarithmic scale. Omental AT (n=7) and subcutaneous AT (n=8).

Discussion

The results of the present study showed that an acute systemic inflammation did not influence adiponectin and leptin synthesis, neither measured as plasma concentrations after vaccination, nor measured as mRNA gene expression in omental or subcutaneous AT after open heart surgery.

When circulating levels of adiponectin were investigated in patients with acute myocardial infarction, a reduction of adiponectin was found to inversely correlate to C-reactive protein with a decrease 24 hrs after myocardial infarction (13). Furthermore, following open heart surgery, plasma levels of leptin do not increase until 24 hrs post-surgery (14). In our study we did not find any differences in plasma levels of adiponectin or leptin up to 24 hrs after vaccination. One possible explanation is that vaccination, that resulted in increased gene expression of TNF in peripheral blood mononuclear cells after four hrs and doubled plasma levels of IL-6 after eight hrs (11), might have been too weak as a model of inflammation. We have previously reported on a very rapid onset of a strong innate immune response in AT following systemic inflammation induced by open heart surgery (12), therefore we investigated adiponectin and leptin on a gene expression level AT after surgery. However, in the present study we could not find any changes in mRNA of these adipokines neither in omental nor in subcutaneous AT after surgery. Possible reasons for this could be either that that we have analyzed the AT gene expression too early or that these adipokines are not key elements in the acute-phase response. Our results do not exclude that adiponectin and leptin levels change later in the inflammatory reaction.

Previously, *in vitro* experiments on human macrophages have demonstrated inhibitory effects of adiponectin on LPS-induced production of TNF, indicating that adiponectin is an important regulator of the immune system (15). However, in that study adiponectin did not affect IL-6 expression in macrophages which indicates that IL-6 may have inhibitory effects on adiponectin in AT (16). Interestingly, we could not find any suppressed gene expression of adiponectin despite a marked upregulation of IL-6 mRNA in AT early after open heart surgery, however, a late effect could not be excluded.

When leptin synthesis has been studied in cultured human subcutaneous adipocytes it has been demonstrated that TNF attenuated mRNA gene expression but in contrast, induced an increased release (8). Furthermore, another *in vitro* experiment by Bruun and coworkers have shown that the proinflammatory cytokines IL-1β and TNF both decreased leptin gene expression and protein production but interestingly IL-1β was found to elicit an early release of leptin (17). The results of these two *in vitro* studies suggest a pre-formed pool of leptin in human AT with a paracrine regulation to which IL-1β and TNF could be important key regulators. However, this pool has not yet been identified and it is not clear whether human adipocytes stimulated *in vitro* adequately represent the situation on a tissue level *in vivo*. In the present study we could not find any influences on plasma levels of leptin after vaccination. Nor after open heart surgery there were any changes in leptin gene expression neither in omental nor in subcutaneous AT.

There are also some recent *in vivo* studies that have focused on both adiponectin and leptin synthesis in relation to acute inflammation. Anderson and coworkers showed that leptin levels increased in plasma after LPS injection but adiponectin levels remained unchanged. On a gene expression level only adiponectin was affected with suppressed expression in subcutaneous AT, whereas there was only a trend towards increased gene expression of leptin (9). The study by Jernås and coworkers showed no effect of inflammation, caused by subarachnoidal haemorrhage, on adiponection and leptin mRNA gene expression in subcutaneous AT (10).

The results of our study support the findings by Jernås and coworkers (10). Furthermore, our results extend previous studies by also analyzing gene expression in omental AT. The reason for the discrepant results is not clear but methodological issues have to be considered. First, the stimulus to inflammation was different between the studies. Anderson and coworkers (9) used LPS whereas we (in the open heart surgery study) and Jernås and coworkers (10) used tissue damage and in our case, possibly also CPB to stimulate innate immunity. All of these are strong stimuli but might activate different subsets of cells, including macrophages in AT. It can be argued that the inflammatory stimulus on AT was similar in our study compared with the study by Anderson and coworkers (9) since we had a similar strong gene expression of IL-6 (12). Also the time-frames were different with the possibility that we, in the open heart surgery study investigated gene expression in AT too early (approximately two hrs) whereas Jernås and coworkers (10) investigated gene expression too late (days). Finally, there is a possibility that the gene expression results in the study by Anderson and coworkers (9) might be false due to up-regulation of β-actin by inflammation rather than down-regulation of adiponectin. In our study, we also tested the results using other house-keeping genes than Cyklophyllin A without changing the negative result (data not shown).

One limitation in the present study may be the low number of patients investigated in the gene expression experiment but we used paired AT biopsies which minimize a possible inter-individual variation. Furthermore, the size of the study group was large enough to demonstrate a very rapid onset of a marked innate immune response in AT following systemic inflammation induced by open heart surgery (12).

Both models of stimulated inflammation used in this study activate inflammation through the nuclear factor-κB pathway (11, 12) and interestingly, none of the models were shown to have any influence on the synthesis of adiponectin or leptin which indicates that the nuclear factor-κB pathway is not involved in the regulation of these two adipokines in an acute-phase response. This is also supported by a recent study by Diez and co-workers who showed that leptin was not found to act as an inflammatory reactant but more as a marker of nutritional status in patients with pneumonia (18).

In conclusion, despite the use of two models of stimulated *in vivo* systemic inflammation we found no evidence of an early regulation of adiponectin and leptin synthesis, indicating that these two adipokines are not key elements in an acute systemic inflammation in humans.

Acknowledgements
We would like to thank Eva Wallgren at the department of Cardiology, Karolinska University Hospital, Solna, for excellent technical assistance.

Grants: This work was supported by grants from the Swedish Heart Lung Foundation.

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Adipose tissue inflammation and coagulation in humans

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska Institutet
offentligen försvaras i Thorax Aula, Karolinska Universitetssjukhuset i Solna,
fredagen den 22 oktober 2010 klockan 9.00
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Stockholm 2010

ABSTRACT

Background

Adipose tissue (AT) is not only a store of energy but an endocrine organ with capacity to produce and release proinflammatory mediators into the circulation. Obesity is an inflammatory disease, with increased circulating levels of interleukin (IL)-6, due to synthesis in AT. As current knowledge regarding AT inflammation, to a great extent relies on studies done in non-stimulated or chronic inflammatory conditions, it is important to add data from human studies, using different models of induced acute systemic inflammation. As obesity is becoming a global disease it is also an increasing risk factor for cardiovascular disease (CVD). CVD events are known complications after surgery and severe infection. The mechanisms behind this increased risk are still poorly understood but an acute systemic inflammation is a common denominator.

Methods and results

Study I: We investigated if a standardised systemic inflammation, induced by a vaccination against Salmonella typhi, would trigger inflammatory gene expression in AT. Healthy volunteers were investigated whereof half of them were vaccinated. Plasma levels of IL-6 increased 8 hrs after vaccination. In peripheral blood mononuclear cells we found an increased tumour necrosis factor gene expression after 4 hrs. In AT there were no differences in gene expression between the two groups.

Study II: Gene expression and production of inflammatory mediators in different AT depots were investigated after open heart surgery. Plasma levels of IL-6 increased 25-fold. In both omental and subcutaneous AT, we found a strong upregulation of nuclear factor-κB regulated genes. Immunohistochemistry (IHC) showed staining for E-selectin associated with a high number of macrophages in close contact with and in the vascular wall. Increased levels of IL-6 were detected in microdialysate from subcutaneous AT.

Study III: Plasminogen activator inhibitor-1 (PAI-1) synthesis in AT was studied after acute systemic inflammation, induced by open heart surgery. Gene expression of PAI-1 increased 27-fold in omental AT and 3-fold in subcutaneous AT. After surgery, IHC staining showed localization of PAI-1 antigen within endothelial cells, in the AT interstitium close to AT vessels and in solitary cells between the adipocytes. The upregulated gene expression and protein synthesis in AT was followed by increased concentrations of PAI-1 antigen in plasma.

Study IV: This was a sub-study of study I and II, with the aim to investigate the effects of an acute systemic inflammation on adiponectin and leptin synthesis. Neither plasma levels of adiponectin nor leptin were changed after vaccination. Gene expression of adiponectin and leptin were unaltered in both omental and subcutaneous AT after surgery.

Conclusion

Vaccination stimulates a mild systemic inflammation but does not trigger proinflammatory gene expression in AT. Open heart surgery induced a strong inflammatory response in both omental and subcutaneous AT including adhesion of macrophages to an activated endothelium and release of IL-6 from AT interstitium. We found no evidence that an acute systemic inflammation could affect synthesis of adiponectin or leptin indicating that these two adipokines are not key elements in the early acute-phase response. There was a markedly increased gene expression and protein synthesis of PAI-1 in human AT after open heart surgery. The increase was most prominent in omental AT. PAI-1 synthesis in AT, following acute systemic inflammation, may be the link between inflammation and impaired fibrinolysis that might explain the increased risk for myocardial infarction after surgery or infection.

ISBN 978-91-7457-037-3